

[illegible]

OF

FOR

INSULIN HOMOLOG POLYPEPTIDE ZINS4

INSULIN HOMOLOG POLYPEPTIDE ZINS4

This application is related to Provisional Application 60/188,544, filed on March 10, 2000. Under 35 U.S.C. § 119(e)(1), this application claims benefit of said Provisional Application.

Proliferation and differentiation of cells within multicellular organisms is controlled by hormones and polypeptide growth factors. These diffusible molecules allow cells to communicate with each other and act in concert to form organs, and to repair and regenerate damaged tissue. Examples of hormones and growth factors include the steroid hormones (e.g., estrogen, testosterone), parathyroid hormone, follicle stimulating hormone, the interleukins, platelet derived growth factor (PDGF), epidermal growth factor (EGF), granulocyte-macrophage colony stimulating factor (GM-CSF), erythropoietin (EPO), insulin and calcitonin.

Hormones and growth factors influence cellular metabolism by binding to receptors. Receptors may be integral membrane proteins that are linked to signaling pathways within the cell, such as second messenger systems. Other classes of receptors are soluble molecules, such as certain transcription factors.

Insulin belongs to a group of protein/polypeptide hormones. Insulin increases the rate of synthesis of glycogen, fatty acids, and proteins and stimulates glycolysis. It also promotes the transport of glucose, some other sugars, and amino acids into muscle and fat cells. The mature form of insulin consists of a 30 amino acid residue B chain, that is at the N-terminus of the propeptide form, and a 21 amino acid residue A chain, that is C-terminal. Proinsulin also contains a connecting peptide, C-peptide, between the B chain and A chain that is cleaved out to form mature insulin. The B chain and A chain are covalently joined by two disulfide bonds. The B-chain, C-peptide, A-chain motif is found in several other proteins including, relaxin (U.S. Patent No. 4,835,251), insulin-like growth factors (IGF) I and II (Bang and Hall, In "Insulin-like Growth Factors", P. N. Schofield (eds.), 151-177, Oxford Univ. Press, Oxford), Leydig factor (Bullesbach et al., *J. Biol. Chem.* 270:16011-15, 1995), Leydig insulin-like peptide (LEY I-L, Burkhardt et al., *Hum. Genet.* 94:91-4, 1994), early placenta

insulin-like factor (EPIL or INSL4; Chassin et al., *Genomics* 29:465-70, 1995), INSL5 (zins3, Conklin et al., *Genomics* 60:50-60, 1999), and INSL6 (zins2, Lok et al., WIPO Publication WO98/05785). Unlike the other members of the insulin superfamily, IGF I and IGF II have D and E domains that are cleaved post-translationally. Cysteines that
 5 are involved in disulfide bonds are conserved in all the members of the family and play a role in the tertiary structure of the molecules.

Growth factors have had an enormous impact on our understanding of and ability to treat metabolic and cellular disorders, discovery of new factors is important. These and other aspects of the invention will become evident upon
 10 reference to the following detailed description of the invention and attached drawings.

SUMMARY OF THE INVENTION

Within one aspect the invention provides an isolated polypeptide comprising amino acid residues 26-52 of SEQ ID NO:2. Within one embodiment the
 15 polypeptide further comprises amino acid residues 119-142 of SEQ ID NO:2. Within a related embodiment the said polypeptide further comprises amino acid residues 55-114 of SEQ ID NO:2. Within another embodiment the polypeptide comprises the amino acid residues 26-142 of SEQ ID NO:2. Within another embodiment polypeptide comprises the amino acid residues 1-142 of SEQ ID NO:2.

20 The invention also provides an isolated polypeptide having an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2, wherein said polypeptide specifically binds with an antibody to which a polypeptide having the amino acid sequence of SEQ ID NO:2 specifically binds. Within one embodiment the sequence that is at least 90% identical to the amino acid
 25 sequence of SEQ ID NO:2. Within another embodiment the isolated polypeptide comprises the sequences of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5. Within yet another embodiment any difference between said amino acid sequence and said corresponding amino acid sequence of SEQ ID NO:2 is due to one or more conservative amino acid substitutions. Within a related embodiment the amino acid
 30 percent identity is determined using a FASTA program with ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=blosum62, with other parameters set as default.

Also provided is an isolated polypeptide comprising the amino acid sequence of SEQ ID NO:2. Additionally, the invention provides an isolated
 35 polypeptide selected from the group consisting of: a) a polypeptide consisting of the sequence of amino acid residues from residue 26 to residue 52 of SEQ ID NO:2;

- b) a polypeptide consisting of the sequence of amino acid residues from residue 26 to residue 53 of SEQ ID NO:2; c) a polypeptide consisting of the sequence of amino acid residues from residue 26 to residue 54 of SEQ ID NO:2; d) a polypeptide consisting of the sequence of amino acid residues from residue 55 to residue 114 of SEQ ID NO:2; e) a polypeptide consisting of the sequence of amino acid residues from residue 55 to residue 115 of SEQ ID NO:2; f) a polypeptide consisting of the sequence of amino acid residues from residue 55 to residue 116 of SEQ ID NO:2; g) a polypeptide consisting of the sequence of amino acid residues from residue 55 to residue 117 of SEQ ID NO:2; h) a polypeptide consisting of the sequence of amino acid residues from residue 55 to residue 118 of SEQ ID NO:2; i) a polypeptide consisting of the sequence of amino acid residues from residue 119 to residue 142 of SEQ ID NO:2; j) a polypeptide consisting of the sequence of amino acid residues from residue 26 to residue 114 of SEQ ID NO:2; k) a polypeptide consisting of the sequence of amino acid residues from residue 26 to residue 118 of SEQ ID NO:2; l) a polypeptide consisting of the sequence of amino acid residues from residue 55 to residue 142 of SEQ ID NO:2; m) a polypeptide consisting of the sequence of amino acid residues from residue 1 to residue 25 of SEQ ID NO:2; n) a polypeptide consisting of the sequence of amino acid residues from residue 1 to residue 52 of SEQ ID NO:2; o) a polypeptide consisting of the sequence of amino acid residues from residue 26 to residue 54 of SEQ ID NO:2; and p) a polypeptide consisting of the sequence of amino acid residues from residue 1 to residue 118 of SEQ ID NO:2.

The invention also provides an isolated protein having: a B chain comprising amino acid residue 26 to amino acid residue 52 of SEQ ID NO:2; and an A chain comprising amino acid residue 119 to amino acid residue 142 of SEQ ID NO:2; wherein the B chain and A chain are joined by inter- and intra-chain disulfide bonds.

Also provided is an isolated polypeptide as described above, further comprising an affinity tag or binding domain.

Within another aspect the invention provides an isolated polynucleotide molecule that encodes a polypeptide as described above. The invention also provides an isolated polynucleotide molecule of SEQ ID NO:1. Additionally the invention provides an isolated polynucleotide selected from the group consisting of: a) a polynucleotide molecule consisting of nucleotides 74-156 of SEQ ID NO:1; b) a polynucleotide consisting of nucleotides 74-159 of SEQ ID NO:1; c) a polynucleotide consisting of nucleotides 74-162 of SEQ ID NO:1; d) a polynucleotide consisting of nucleotides 163-342 of SEQ ID NO:1; e) a polynucleotide consisting of nucleotides 163-345 of SEQ ID NO:1; f) a polynucleotide consisting of nucleotides 163-348 of SEQ ID NO:1; g) a polynucleotide consisting of nucleotides 163-351 of SEQ ID NO:1; h) a polynucleotide consisting of nucleotides 163-354 of SEQ ID NO:1; i) a

polynucleotide consisting of nucleotides 355-426 of SEQ ID NO:1; j) a polynucleotide consisting of nucleotides 1-73 of SEQ ID NO:1; k) a polynucleotide consisting of nucleotides 1-162 of SEQ ID NO:1; l) a polynucleotide consisting of nucleotides 1-342 of SEQ ID NO:1; m) a polynucleotide consisting of nucleotides 74-342 of SEQ ID NO:1; n) a polynucleotide consisting of nucleotides 74-345 of SEQ ID NO:1; o) a polynucleotide consisting of nucleotides 74-348 of SEQ ID NO:1; p) a polynucleotide consisting of nucleotides 74-351 of SEQ ID NO:1; and q) a polynucleotide consisting of nucleotides 74-354 of SEQ ID NO:1.

The invention also provides an expression vector comprising the following operably linked elements: a transcription promoter; a DNA molecule that encodes a polypeptide as described above; and a transcription terminator.

Additionally the invention provides cultured cell into which has been introduced an expression vector comprising the following operably linked elements: a transcription promoter; a polynucleotide molecule that encodes a polypeptide according to claim 1; and a transcription terminator, wherein said cultured cell expresses said polypeptide encoded by said polynucleotide segment. Within one embodiment the cell further comprises a second expression vector comprising the following operably linked elements: a transcriptional promoter; a DNA sequence encoding a prohormone convertase; and a transcriptional terminator. Within yet another embodiment the prohormone convertase is selected from the group consisting of prohormone convertase 1/3, prohormone convertase 2, prohormone convertase 4, PACE, PACE4, furin, and kex2.

The invention also provides a method of producing a protein comprising: culturing a cell into which has been introduced an expression vector comprising the following operably linked elements: a transcription promoter; a polynucleotide molecule that encodes a polypeptide as described above; and a transcription terminator; whereby said cell expresses said polypeptide encoded by said polynucleotide segment; and recovering said expressed protein.

The invention also provides a fusion protein, comprising the polypeptide as described above.

Within another aspect the invention provides an antibody or antibody fragment that specifically binds to a polypeptide as described above. Within one embodiment the antibody is selected from the group consisting of: a) polyclonal antibody; b) murine monoclonal antibody; c) humanized antibody derived from b); and d) human monoclonal antibody. Within another embodiment the antibody fragment is selected from the group consisting of F(ab'), F(ab), Fab', Fab, Fv, scFv, and

minimal recognition unit. The invention also provides an anti-idiotypic antibody that specifically binds to an antibody as described above.

The invention also provides a polypeptide as described above, in combination with a pharmaceutically acceptable vehicle.

5 The invention further provides a method for producing, from a genomic DNA source, a contiguous polypeptide encoding polynucleotide sequence free from introns comprising the steps of: a) amplifying exon segments using exon specific primer pairs having a type II-S restriction endonuclease site near the primer 5' terminus; b) digest the amplified exon segments with the type II-S restriction endonuclease to
10 produce cohesive overhangs; and c) ligate digested exon segments. Within one embodiment the genomic DNA source is purified genomic DNA, a genomic clone, a BAC, a PAC, genomic library, or chromosomal DNA clone.

DETAILED DESCRIPTION OF THE INVENTION

15 Prior to setting forth the invention in detail, it may be helpful to the understanding thereof to define the following terms:

The term "affinity tag" is used herein to denote a polypeptide segment that can be attached to a second polypeptide to provide for purification or detection of the second polypeptide or provide sites for attachment of the second polypeptide to a
20 substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A (Nilsson et al., *EMBO J.* 4:1075, 1985; Nilsson et al., *Methods Enzymol.* 198:3, 1991), glutathione S transferase (Smith and Johnson, *Gene* 67:31, 1988), Glu-Glu affinity tag (Grussenmeyer et al., *Proc. Natl. Acad. Sci. USA* 82:7952-4,
25 1985), substance P, Flag™ peptide (Hopp et al., *Biotechnology* 6:1204-10, 1988), streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general, Ford et al., *Protein Expression and Purification* 2: 95-107, 1991. DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

30 The term "allelic variant" is used herein to denote any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequence. The term allelic variant
35 is also used herein to denote a protein encoded by an allelic variant of a gene.

The terms "amino-terminal" (N-terminal) and "carboxyl-terminal" (C-terminal) are used herein to denote positions within polypeptides. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a polypeptide is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete polypeptide.

The term "complement/anti-complement pair" denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions. For instance, biotin and avidin (or streptavidin) are prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of the complement/anti-complement pair is desirable, the complement/anti-complement pair preferably has a binding affinity of $<10^9 \text{ M}^{-1}$.

The term "complements of a polynucleotide molecule" denotes a polynucleotide molecule having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "contig" denotes a polynucleotide that has a contiguous stretch of identical or complementary sequence to another polynucleotide. Contiguous sequences are said to "overlap" a given stretch of polynucleotide sequence either in their entirety or along a partial stretch of the polynucleotide. For example, representative contigs to the polynucleotide sequence 5'-ATGGCTTAGCTT-3' are 5'-TAGCTTgagtct-3' and 3'-gtcgacTACCGA-5'.

The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and GAC triplets each encode Asp).

The term "expression vector" is used to denote a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments include promoter and terminator sequences, and may also include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, etc. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

5

15

20

25

organism. Paralogs are believed to arise through gene duplication. For example, α -globin, β -globin, and myoglobin are paralogs of each other.

30

35

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides".

The term "promoter" is used herein for its art-recognized meaning to denote a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

A "protein" is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule (i.e., a ligand) and mediates the effect of the ligand on the cell. Membrane-bound receptors are characterized by a multi-peptide structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. Binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell. This interaction in turn leads to an alteration in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids. In general, receptors can be membrane bound, cytosolic or nuclear; monomeric (e.g., thyroid stimulating hormone receptor, beta-adrenergic receptor) or multimeric (e.g., PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor).

The term "secretory signal sequence" denotes a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

The term "splice variant" is used herein to denote alternative forms of RNA transcribed from a gene. Splice variation arises naturally through use of alternative splicing sites within a transcribed RNA molecule, or less commonly between separately transcribed RNA molecules, and may result in several mRNAs transcribed from the same gene. Splice variants may encode polypeptides having altered amino acid sequence. The term splice variant is also used herein to denote a protein encoded by a splice variant of an mRNA transcribed from a gene.

Molecular weights and lengths of polymers determined by imprecise analytical methods (e.g., gel electrophoresis) will be understood to be approximate values. When such a value is expressed as "about" X or "approximately" X, the stated value of X will be understood to be accurate to $\pm 10\%$.

All references cited herein are incorporated by reference in their entirety.

The present invention is based in part upon the discovery of a novel DNA sequence that encodes a novel polypeptide having homology to the relaxin family. The polypeptide has been designated zins4.

A genomic DNA sequence was discovered and exons were predicted to code for a secreted full-length protein. The nucleotide sequence encoding a full-length zins4 polypeptide is described in SEQ ID NO:1, and its deduced amino acid sequence is described in SEQ ID NO:2. The mature polypeptide has homology to the relaxin family. The zins4 polypeptide of SEQ ID NO:2 shares 28% identity over a 117 amino acid residue overlap with spiny dogfish relaxin (RELX_SQUAC, Bullesbach et al., *Eur. J. Biochem.* 161:335-41, 1986); and 20.6% identity over a 194 amino acid residue overlap with human prorelaxin H2 precursor (REL2_Human, Hudson et al., *EMBO J.* 3:2333-39, 1984). Percent identity was determined using the Smith-Waterman algorithm (Smith and Waterman, *J. Mol. Biol.* 147:195-7, 1981; and Pearson, *Genomics* 11:635-50, 1991).

Within the relaxin family the cysteine motif is highly conserved in the B and A chains, where the B chain motif can be represented as LCGX{10}C, where X{ } is the number of amino acid residues. X is any amino acid residue except cysteine. The A chain motif is CCX{3}CX{8}C (SEQ ID NO:4), where X{ } is the number of any amino acid residues. X is any amino acid residue except cysteine. The B chain also contains a conserved motif RXXXR (SEQ ID NO:5). X represents any amino acid residue except cysteine. The two arginine residues are necessary for relaxin binding to cells and tissues. If either arginine is changed synthetically, there is no competition for relaxin binding.

The DNA sequence encoding the zins4 polypeptide revealed that the predicted amino acid sequence contained the B chain-C peptide-A chain motif found in

- the relaxins and insulin. Preprorelaxin and preproinsulin both have a signal sequence, followed by the B chain, C peptide, and then the A chain. The mature relaxins and insulin have the signal peptide and C peptide removed, with the B and A chains joined by both inter- and intra-chain disulfide bonds (James et al., *Nature* 267:544-546, 1977).
- 5 Sequence analysis indicates that the human polypeptide sequence (SEQ ID NO: 2) is structurally equivalent to other members of the family.

- Processing of the protein involves cleavage at the C-terminus of the signal peptide, and, based on predicted structural homology with other mature members of the insulin family, a cleavage at the C-terminus of the B chain and at the N-terminus
- 10 of the A chain, resulting in removal of the C-peptide. Analysis of the zins4 polypeptide of SEQ ID NO:2 with other known members of the insulin family suggests a signal peptide cleavage site in the region of amino acid residue 25 (Ala) of SEQ ID NO:2. Cleavage at the C-terminus of the B chain is predicted to be at the C-terminal of amino acid residue 53 (Arg) or residue 54 (Arg) followed by cleavage of the Arg residues by
- 15 carboxypeptidase to leave amino acid residue 52 (Trp) as the C-terminal amino acid residue. Cleavage sites resulting in the N-terminus of the A chain are suggested in the region of amino acid residue 115 (Arg) to 118 (Arg). Cleavage is predicted to be after the C-terminus of amino acid residue 118 (Arg) leaving amino acid residue 119 (Asp) as the N-terminal amino acid residue of the A chain. The C-terminal amino acid is
- 20 residue 142 (Cys). The cleavage site at the junction of the C-peptide and A chain is highly conserved, occurring after Arg-X-X-Arg (wherein X is any amino acid residue), Arg-Arg or Lys-Arg; however, the cleavage sites at the junction of the signal sequence and B chain, and at the junction of the B chain and C-peptide, do not maintain a similarly high degree of conservation within the insulin family.

- 25 Based on the predicted sites, the amino acid sequence of mature zins4 includes a B chain having the sequence of SEQ ID NO:2 from amino acid residue 26 (Arg) to amino acid residue 52 (Trp), and an A chain having the sequence of SEQ ID NO:2 from amino acid residue 119 (Asp) to amino acid residue 142 (Cys).

- The enzymology of proinsulin conversion suggests that prohormone
- 30 convertase 1/3 (PC1/3), or prohormone convertase 2 (PC2), cleave zins4 primarily at the B chain-C-peptide junction. Following cleavage at this site, it is predicted that a carboxypeptidase will remove the resulting C-terminal arginine residues and leave amino acid residue 30 (Trp) of SEQ ID NO:2 as the C-terminal residue of the B-chain in the mature hormone. Members of the furin family of convertases, furin, PACE,
- 35 PACE4, and prohormone convertase 4 (PC4) cleave preferentially at the C-peptide-A-chain junction. Also useful is the yeast dibasic-specific endoprotease Kex2. Co-expression of zins4 with an appropriate member of the furin family of prohormone

5 Within the relaxin proteins there is a highly conserved region in the B chain represented by the motif RXXXR (amino acid residues 37-41 of SEQ ID NO:2) and the motif LCGX{10}C (amino acid residues 34-47 of SEQ ID NO:2), where X{ } is the number of any amino acid residues, and X is any amino acid residue except cysteine. The A chain contains a conserved motif CCX{3}CX{8}C (amino acid residues 128-142 of SEQ ID NO:2), where X{ } is the number of any amino acid residues, and X is any amino acid residue except cysteine.

Chromosomal localization of *zins4* has been made to chromosome 19p13.11. As used herein *zins4* designates nucleotide sequence or related matters and *zins4* designates polypeptide sequences.

The present invention also provides polynucleotide molecules, including DNA and RNA molecules, that encode the zins4 polypeptides disclosed herein. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. SEQ ID NO:6 is a degenerate DNA sequence that encompasses all DNAs that encode the zins4 polypeptide of SEQ ID NO:2. Those skilled in the art will recognize that the degenerate sequence of SEQ ID NO:6 also provides all RNA sequences encoding polypeptides of SEQ ID NO:2 by substituting U for T. Thus, zins4 polypeptide-encoding polynucleotides comprising nucleotide 1 to nucleotide 429 of SEQ ID NO:1 and their RNA equivalents are contemplated by the present invention. Table 1 sets forth the one-letter codes used within SEQ ID NO:6 to denote degenerate nucleotide positions. "Resolutions" are the nucleotides denoted by a code letter. "Complement" indicates the code for the complementary nucleotide(s). For example, the code Y denotes either C or T, and its complement R denotes A or G, A being complementary to T, and G being complementary to C.

TABLE 1

Nucleotide	Resolution	Complement	Resolution
A	A	T	T
C	C	G	G
G	G	C	C
T	T	A	A
R	A G	Y	C T
Y	C T	R	A G
M	A C	K	G T
K	G T	M	A C
S	C G	S	C G
W	A T	W	A T
H	A C T	D	A G T
B	C G T	V	A C G
V	A C G	B	C G T
D	A G T	H	A C T
N	A C G T	N	A C G T

The degenerate codons used in SEQ ID NO:6, encompassing all possible
5 codons for a given amino acid, are set forth in Table 2.

TABLE 2

Amino Acid	One Letter Code	Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	ACN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAY
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn Asp	B		RAY
Glu Gln	Z		SAR
Any	X		NNN

093407-0000
105020-2207260

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding each amino acid. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by the degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequence of SEQ ID NO:2. Variant sequences can be readily tested for functionality as described herein.

One of ordinary skill in the art will also appreciate that different species can exhibit "preferential codon usage." In general, see, Grantham, et al., *Nuc. Acids Res.* 8:1893-912, 1980; Haas, et al. *Curr. Biol.* 6:315-24, 1996; Wain-Hobson, et al., *Gene* 13:355-64, 1981; Grosjean and Fiers, *Gene* 18:199-209, 1982; Holm, *Nuc. Acids Res.* 14:3075-87, 1986; Ikemura, *J. Mol. Biol.* 158:573-97, 1982. As used herein, the term "preferential codon usage" or "preferential codons" is a term of art referring to protein translation codons that are most frequently used in cells of a certain species, thus favoring one or a few representatives of the possible codons encoding each amino acid (See Table 2). For example, the amino acid threonine (Thr) may be encoded by ACA, ACC, ACG, or ACT, but in mammalian cells ACC is the most commonly used codon; in other species, for example, insect cells, yeast, viruses or bacteria, different Thr codons may be preferential. Preferential codons for a particular species can be introduced into the polynucleotides of the present invention by a variety of methods known in the art. Introduction of preferential codon sequences into recombinant DNA can, for example, enhance production of the protein by making protein translation more efficient within a particular cell type or species. Therefore, the degenerate codon sequence disclosed in SEQ ID NO:6 serves as a template for optimizing expression of polynucleotides in various cell types and species commonly used in the art and disclosed herein. Sequences containing preferential codons can be tested and optimized for expression in various species, and tested for functionality as disclosed herein.

Within preferred embodiments of the invention the isolated polynucleotides will hybridize to similar sized regions of SEQ ID NO:1, or a sequence complementary thereto, under stringent conditions. In general, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Numerous equations for calculating T_m are known in the art, and are

specific for DNA, RNA and DNA-RNA hybrids and polynucleotide probe sequences of varying length (see, for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition (Cold Spring Harbor Press 1989); Ausubel *et al.*, (eds.), *Current Protocols in Molecular Biology* (John Wiley and Sons, Inc. 1987); Berger and Kimmel (eds.), *Guide to Molecular Cloning Techniques*, (Academic Press, Inc. 1987); and Wetmur, *Crit. Rev. Biochem. Mol. Biol.* 26:227 (1990)). Sequence analysis software such as OLIGO 6.0 (LSR; Long Lake, MN) and *Primer Premier 4.0* (Premier Biosoft International; Palo Alto, CA), as well as sites on the Internet, are available tools for analyzing a given sequence and calculating T_m based on user defined criteria. Such programs can also analyze a given sequence under defined conditions and identify suitable probe sequences. Typically, hybridization of longer polynucleotide sequences, >50 base pairs, is performed at temperatures of about 20-25°C below the calculated T_m . For smaller probes, <50 base pairs, hybridization is typically carried out at the T_m or 5-10°C below. This allows for the maximum rate of hybridization for DNA-DNA and DNA-RNA hybrids. Higher degrees of stringency at lower temperatures can be achieved with the addition of formamide which reduces the T_m of the hybrid about 1°C for each 1% formamide in the buffer solution. Suitable stringent hybridization conditions are equivalent to about a 5 h to overnight incubation at about 42°C in a solution comprising: about 40-50% formamide, up to about 6X SSC, about 5X Denhardt's solution, zero up to about 10% dextran sulfate, and about 10-20 µg/ml denatured commercially-available carrier DNA. Generally, such stringent conditions include temperatures of 20-70°C and a hybridization buffer containing up to 6x SSC and 0-50% formamide; hybridization is then followed by washing filters in up to about 2X SSC. For example, a suitable wash stringency is equivalent to 0.1X SSC to 2X SSC, 0.1% SDS, at 55°C to 65°C. Different degrees of stringency can be used during hybridization and washing to achieve maximum specific binding to the target sequence. Typically, the washes following hybridization are performed at increasing degrees of stringency to remove non-hybridized polynucleotide probes from hybridized complexes. Stringent hybridization and wash conditions depend on the length of the probe, reflected in the T_m , hybridization and wash solutions used, and are routinely determined empirically by one of skill in the art.

As previously noted, the isolated polynucleotides of the present invention include DNA and RNA. Methods for preparing DNA and RNA are well known in the art and are discussed herein. *Zins4* is representative of an emerging class of genes that were first discovered through interrogation of genomic databases. The absence of information pointing to a source of mRNA template makes it difficult to employ conventional means to isolate a full-length cDNA for these genes.

Additionally, many of these genes are not represented in current EST databases suggesting that they are either expressed at very low levels or by limited cell-types or at restricted times.

Isolation of a cDNA encoding the full-length polypeptide is a necessary first step for producing recombinant protein. While it is possible for some eukaryotic expression system to produce recombinant proteins encoded by introduced genomic sequences, many genes contain multiple introns whose collective length renders the expression unit too large to be efficiently inserted in most plasmid-based expression vectors. Another challenge posed by genomic sequence is the possible presence of repeated elements within introns. These elements may promote plasmid-instability while the expression vector is being propagated through the bacterial host. The presence of intronic sequences within the expression cassette leaves open the possibility for the recipient mammalian host cell to use cryptic splice donor and acceptor sites within the introns. Such alternative splice sites may be natural to the gene or may be artifactual to the expression host cell. The use of these cryptic splice sites by the host cell could lead to the production of a different recombinant polypeptide than intended. The splicing mechanisms of mammalian, yeast or insect cells may also be sufficiently different from one another to preclude accurate or efficient splicing of some mRNAs transcribed by heterologous genes. Finally, the lack of mRNA splicing in bacterial host cells necessitates the removal of all intronic sequence within the expression unit for the production of recombinant protein in such systems. Hence, there is a need for a rapid and efficient method to convert gene sequence to a contiguous polypeptide coding sequence that is free of introns.

The polymerase chain reaction (PCR) provides a convenient method for isolating defined DNA segments through the use of sequence specific pairs of oligonucleotide primers. While the use of PCR and exon-specific primers would enable the isolation of exon gene segments, rejoining the resulting exon segments to produce a contiguous sequence encoding the polypeptide of interest is difficult. To overcome this problem, oligonucleotide primers can be designed that incorporate the polynucleotide sequence of a type II restriction endonuclease cleavage site near the 5' end of each member of the primer pair. The resulting PCR product derived from these primers would incorporate the restriction endonuclease site at its terminus, which upon digestion with the corresponding type II restriction enzyme, would produce suitable cohesive overhangs to promote efficient ligation in the presence of DNA ligase. This method, however, cannot be employed to ligate exon segments to produce a contiguous polypeptide coding sequence. Exon segments ligated together in this manner would contain a foreign restriction endonuclease recognition sequence between ligated

At high DNA and DNA ligase concentrations, it is possible to ligate blunt-ended DNA segments together. The so called blunt-end ligation reaction would enable the ligation of PCR generated exon segments without the need to incorporate restriction endonuclease sites to the primers and thereby the ligated products would be free of foreign sequences. This approach, however, has severe limitations. Since the blunt-end ligation reaction does not make use of defined cohesive ends, the number of combinations and permutations of incorrect ligation products increases exponentially with the number of exons to be ligated, rendering this method impractical for general use.

This method can be used to convert *zins4* genomic DNA into a contiguous polypeptide coding sequence (cPCS). The human zcytor-1 gene (Sprecher et al., *Biochem. Biophys. Res. Comm.* 246:82-90, 1998; Baumgartner et al., U.S. Patent Number 5,792,850) is located 2 kb from the *zins4* locus. Zcytor-1 gene primers were used to isolate a BAC clone containing the gene encoding human *zins4*. A "Down to the Well" human BAC II DNA library (Genome Systems Inc, St. Louis, Missouri) was screened by PCR according to the manufacturer's instructions using *zins4*-specific sense

and antisense primers ZC9736 (SEQ ID NO:7) and ZC9740 (SEQ ID NO:8). A positive genomic BAC clone (328h12) containing the *zins4* gene was identified. DNA from clone 328h12 was prepared for use as a PCR template using an AutoGen 740 automated DNA extraction system (AutoGen, Framingham, MA) in accordance with the manufacturer's instructions.

Zins4 contains two exons. The *zins4* exon 1 DNA segment is isolated using sense oligonucleotide primer (SEQ ID NO:9); and antisense oligonucleotide primer (SEQ ID NO:10), respectively. The *zins4* exon 2 DNA segment is isolated using sense oligonucleotide primer (SEQ ID NO: 11) and antisense oligonucleotide primer (SEQ ID NO:12), respectively. A PCR reaction mix is prepared as follows: to a final volume of 100 µl is added 10 µl 10X native PFU DNA polymerase buffer (Stratagene, La Jolla, CA); 1 µl 20 mM dNTP mix; 30 pmoles each of sense and antisense primers; 10 ng of 218h12 BAC clone DNA template; and 2 units of native PFU DNA polymerase (Stratagene). DNA amplification is carried out as follows: 1 cycle (95°C for 1 minute), followed by 3 cycle of (95°C for 10 seconds; 45°C for 10 seconds; 72°C for 2 minutes) followed by 17 cycles of (95°C for 10 seconds; 60°C for 10 seconds; 72°C for 2 minutes), followed by a further 5 minute extension at 72°C. The amplified *zins4* exon products are extracted with phenol and chloroform and are precipitated with ethanol. The PCR products are digested with the type-IIS restriction endonuclease, *Eco31I* (MBI Fermentas, Amherst, NY) according to manufacturer's instruction. The digested PCR product is purified using a Gel Extraction Kit (Qiagen, Chatsworth, CA) according to manufacturer's instructions.

Ligation of *zins4* exons into *EcoR*-1 and *Xba*-1 digested mammalian expression vector pSAM-8 is carried out in a 5 µl ligation reaction consisted of: 1 µl vector (40 ng); 0.5 µl 10X Promega ligase buffer (Promega Life Science, Madison, WI), 0.5 µl 10 mM ATP, 1 µl gel purified *Eco31I* digested *zins4* exon 1 (~10 ng); 1 µl gel purified *Eco31I* digested *zins4* exon 2 (~10 ng); and 0.5 µl (15 units/µl) T4 DNA Ligase (Promega Life Science). The ligation reaction is carried at 6°C for 2 hrs; 8°C for 2 hrs; 10°C for 2 hrs; 12.5°C for 2 hrs; 15°C for 2 hrs; followed by an overnight incubation at 10°C overnight. One µl of the ligation mixture is used to transform MAX efficiency DH10B competent cells (Life Technologies, Rockville, MD). Transformants are selected by plating onto Ampicillin LB agar plates. The resulting clone carrying the *zins4* cPCS is confirmed by DNA sequence analysis.

RNA may also be isolated from a tissue or cell that produces large amounts of *zins4* RNA. Such tissues and cells are identified by using the sequence information provide herein in combination with Northern blotting (Thomas, *Proc. Natl. Acad. Sci. USA* 77:5201, 1980). Total RNA can be prepared using guanidinium

09341077 020904

isothiocyanate extraction followed by isolation by centrifugation in a CsCl gradient (Chirgwin et al., *Biochemistry* 18:52-94, 1979). Poly (A)⁺ RNA is prepared from total RNA using the method of Aviv and Leder (*Proc. Natl. Acad. Sci. USA* 69:1408-12, 1972). Complementary DNA (cDNA) is prepared from poly(A)⁺ RNA using known methods. In the alternative, genomic DNA can be isolated. Polynucleotides encoding zins4 polypeptides are then identified and isolated by, for example, hybridization or PCR. A full-length clone encoding zins4 can be obtained by conventional cloning procedures. Complementary DNA (cDNA) clones are preferred, although for some applications (e.g., expression in transgenic animals) it may be preferable to use a genomic clone, or to modify a cDNA clone to include at least one genomic intron. Methods for preparing cDNA and genomic clones are well known and within the level of ordinary skill in the art, and include the use of the sequence disclosed herein, or parts thereof, for probing or priming a library. Expression libraries can be probed with antibodies to zins4, receptor fragments, or other specific binding partners.

The polynucleotides of the present invention can also be synthesized using DNA synthesis machines. If chemically synthesized double stranded DNA is required for an application such as the synthesis of a DNA or a DNA fragment, then each complementary strand is made separately, for example via the phosphoramidite method known in the art. The production of short polynucleotides (60 to 80 bp) is technically straightforward and can be accomplished by synthesizing the complementary strands and then annealing them. However, for producing longer polynucleotides (longer than about 300 bp), special strategies are usually employed. For example, synthetic DNAs (double-stranded) are assembled in modular form from single-stranded fragments that are from 20 to 100 nucleotides in length. One method for building a synthetic DNA involves producing a set of overlapping, complementary oligonucleotides. Each internal section of the DNA has complementary 3' and 5' terminal extensions designed to base pair precisely with an adjacent section. After the DNA is assembled, the process is completed by ligating the nicks along the backbones of the two strands. In addition to the protein coding sequence, synthetic DNAs can be designed with terminal sequences that facilitate insertion into a restriction endonuclease site of a cloning vector. Alternative ways to prepare a full-length DNA are also known in the art. See Glick and Pasternak, Molecular Biotechnology, Principles & Applications of Recombinant DNA, (ASM Press, Washington, D.C. 1994); Itakura et al., Annu. Rev. Biochem. 53: 323-56, 1984 and Climie et al., *Proc. Natl. Acad. Sci. USA* 87:633-7, 1990.

The present invention further provides counterpart polypeptides and polynucleotides from other species (orthologs). These species include, but are not

0938407-026994
FILED 4-20-90

limited to mammalian, avian, amphibian, reptile, fish, insect and other vertebrate and invertebrate species. Of particular interest are zins4 polypeptides from other mammalian species, including murine, porcine, ovine, bovine, canine, feline, equine, and other primate polypeptides. Orthologs of human zins4 can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses zins4 as disclosed herein. Suitable sources of mRNA can be identified by probing Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line. A zins4-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequences. A cDNA can also be cloned using the polymerase chain reaction, or PCR (Mullis, U.S. Patent No. 4,683,202), using primers designed from the representative human zins4 sequence disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to zins4 polypeptide. Similar techniques can also be applied to the isolation of genomic clones.

Those skilled in the art will recognize that the sequence disclosed in SEQ ID NO:1 represents a single allele of human zins4 and that allelic variation and alternative splicing are expected to occur. Allelic variants of this sequence can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Allelic variants of the DNA sequence shown in SEQ ID NO:1, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NO:2. cDNAs generated from alternatively spliced mRNAs, which retain the properties of the zins4 polypeptide are included within the scope of the present invention, as are polypeptides encoded by such cDNAs and mRNAs. Allelic variants and splice variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals or tissues according to standard procedures known in the art.

The present invention also provides isolated zins4 polypeptides that are substantially similar to the polypeptides of SEQ ID NO:2 and their orthologs. The term "substantially similar" is used herein to denote polypeptides having 70%, preferably 75%, more preferably at least 80%, sequence identity to the sequences shown in SEQ ID NO:2 or their orthologs. Such polypeptides will more preferably be at least 90% identical, and most preferably 95% or more identical to SEQ ID NO:2 or its orthologs.)

Percent sequence identity is determined by conventional methods. See, for example, Altschul et al., *Bull. Math. Bio.* 48: 603-16, 1986 and Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915-9, 1992. Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "blosum 62" scoring matrix of Henikoff and Henikoff (*supra.*) as shown in Table 3 (amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as:

$$\frac{\text{Total number of identical matches}}{[\text{length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences}]} \times 100$$

106020 2078200

Table 3[illegible]

Sequence identity of polynucleotide molecules is determined by similar methods using a ratio as disclosed above.

Those skilled in the art appreciate that there are many established algorithms available to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson and Lipman is a suitable protein alignment method for
 5 examining the level of identity shared by an amino acid sequence disclosed herein and the amino acid sequence of a putative variant zins4. The FASTA algorithm is described by Pearson and Lipman, *Proc. Nat'l Acad. Sci. USA* 85:2444, 1988, and by Pearson, *Meth. Enzymol.* 183:63, 1990.

10 Briefly, FASTA first characterizes sequence similarity by identifying regions shared by the query sequence (e.g., SEQ ID NO:2) and a test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions, or deletions. The ten regions with the highest density of identities are then rescored by
 15 comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed" to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence and the ktup value), then the trimmed initial regions are examined to
 20 determine whether the regions can be joined to form an approximate alignment with gaps. Finally, the highest scoring regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch-Sellers algorithm (Needleman and Wunsch, *J. Mol. Biol.* 48:444, 1970; Sellers, *SIAM J. Appl. Math.* 26:787, 1974), which allows for amino acid insertions and deletions. Preferred parameters for FASTA
 25 analysis are: ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=BLOSUM62. These parameters can be introduced into a FASTA program by modifying the scoring matrix file ("SMATRIX"), as explained in Appendix 2 of Pearson, *Meth. Enzymol.* 183:63, 1990.

FASTA can also be used to determine the sequence identity of nucleic
 30 acid molecules using a ratio as disclosed above. For nucleotide sequence comparisons, the ktup value can range between one to six, preferably from three to six, most preferably three, with other parameters set as default.

The BLOSUM62 table (Table 3) is an amino acid substitution matrix derived from about 2,000 local multiple alignments of protein sequence segments,
 35 representing highly conserved regions of more than 500 groups of related proteins (Henikoff and Henikoff, *Proc. Nat'l Acad. Sci. USA* 89:10915, 1992). Accordingly, the BLOSUM62 substitution frequencies can be used to define conservative amino acid

substitutions that may be introduced into the amino acid sequences of the present invention. Although it is possible to design amino acid substitutions based solely upon chemical properties (as discussed below), the language "conservative amino acid substitution" preferably refers to a substitution represented by a BLOSUM62 value of greater than -1. For example, an amino acid substitution is conservative if the substitution is characterized by a BLOSUM62 value of 0, 1, 2, or 3. According to this system, preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 1 (e.g., 1, 2 or 3), while more preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 2 (e.g., 2 or 3).

Variant zins4 polypeptides or substantially homologous zins4 polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 4) and other substitutions that do not significantly affect the folding or activity of the polypeptide; small deletions, typically of one to about 30 amino acids; and amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or an affinity tag. The present invention thus includes polypeptides of from about 14 to about 500 amino acid residues that comprise a sequence that is at least 80%, preferably at least 90%, and more preferably 95% or more identical to the corresponding region of SEQ ID NO:2.

1065000 2207860

Table 4Conservative amino acid substitutions

5		
	Basic:	arginine
		lysine
		histidine
	Acidic:	glutamic acid
10		aspartic acid
	Polar:	glutamine
		asparagine
	Hydrophobic:	leucine
		isoleucine
15		valine
	Aromatic:	phenylalanine
		tryptophan
		tyrosine
	Small:	glycine
20		alanine
		serine
		threonine
		methionine

25 The proteins of the present invention can also comprise non-naturally occurring amino acid residues. Non-naturally occurring amino acids include, without limitation, *trans*-3-methylproline, 2,4-methanoproline, *cis*-4-hydroxyproline, *trans*-4-hydroxyproline, *N*-methylglycine, *allo*-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipercolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, 3,3-dimethylproline, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluorophenylalanine. Several methods are known in the art for

30 incorporating non-naturally occurring amino acid residues into proteins. For example,

an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is carried out in a cell-free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson et al., *J. Am. Chem. Soc.* 113:2722, 1991; Ellman et al., *Methods Enzymol.* 202:301, 1991; Chung et al., *Science* 259:806-9, 1993; and Chung et al., *Proc. Natl. Acad. Sci. USA* 90:10145-9, 1993). In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti et al., *J. Biol. Chem.* 271:19991-8, 1996). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (e.g., phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (e.g., 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide et al., *Biochem.* 33:7470-6, 1994. Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, *Protein Sci.* 2:395-403, 1993).

A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, non-naturally occurring amino acids, and unnatural amino acids may be substituted for zins4 amino acid residues.

Essential amino acids in the polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244: 1081-5, 1989; Bass et al., *Proc. Natl. Acad. Sci. USA* 88:4498-502, 1991). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity as disclosed below to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton et al., *J. Biol. Chem.* 271:4699-708, 1996. Sites of ligand-receptor or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., *Science* 255:306-12, 1992; Smith et al., *J. Mol. Biol.* 224:899-904, 1992; Wlodaver et al., *FEBS Lett.* 309:59-64, 1992. The identities of essential amino acids can also be inferred from analysis of homologies with related polypeptide sequences or proteins.

Determination of amino acid residues that are within regions or domains that are critical to maintaining structural integrity can be determined. Within these regions one can determine specific residues that will be more or less tolerant of change and maintain the overall tertiary structure of the molecule. Methods for analyzing sequence structure include, but are not limited to, alignment of multiple sequences with high amino acid or nucleotide identity and computer analysis using available software (e.g., the Insight II[®] viewer and homology modeling tools; MSI, San Diego, CA), secondary structure propensities, binary patterns, complementary packing and buried polar interactions (Barton, *Current Opin. Struct. Biol.* 5:372-376, 1995 and Cordes et al., *Current Opin. Struct. Biol.* 6:3-10, 1996). In general, when designing modifications to molecules or identifying specific fragments determination of structure will be accompanied by evaluating activity of modified molecules.

Amino acid sequence changes are made in zins4 polypeptides so as to minimize disruption of higher order structure essential to biological activity. For example, when the zins4 polypeptide comprises one or more conserved structures, changes in amino acid residues will be made so as not to disrupt the structures and other components of the molecule where changes in conformation abate some critical function, for example, binding of the molecule to its binding partners. The effects of amino acid sequence changes can be predicted by, for example, computer modeling as disclosed herein or determined by analysis of crystal structure (see, e.g., Laphorn et al., *Nat. Struct. Biol.* 2:266-268, 1995). Other techniques that are well known in the art compare folding of a variant protein to a standard molecule (e.g., the native protein). For example, comparison of the cysteine pattern in a variant and standard molecules can be made. Mass spectrometry and chemical modification using reduction and alkylation provide methods for determining cysteine residues which are associated with disulfide bonds or are free of such associations (Bean et al., *Anal. Biochem.* 201:216-226, 1992; Gray, *Protein Sci.* 2:1732-1748, 1993; and Patterson et al., *Anal. Chem.* 66:3727-3732, 1994). It is generally believed that if a modified molecule does not have the same disulfide bonding pattern as the standard molecule folding would be affected. Another well known and accepted method for measuring folding is circular dichroism (CD). Measuring and comparing the CD spectra generated by a modified molecule and standard molecule is routine (Johnson, *Proteins* 7:205-214, 1990). Crystallography is another well known method for analyzing folding and structure. Nuclear magnetic resonance (NMR), digestive peptide mapping and epitope mapping are also known methods for analyzing folding and structural similarities between proteins and polypeptides (Schaanan et al., *Science* 257:961-964, 1992).

A Hopp/Woods hydrophilicity profile of the zins4 protein sequence as shown in SEQ ID NO:2 can be generated (Hopp et al., *Proc. Natl. Acad. Sci.* 78:3824-3828, 1981; Hopp, *J. Immun. Meth.* 88:1-18, 1986 and Triquier et al., *Protein Engineering* 11:153-169, 1998). Those skilled in the art will recognize that hydrophilicity or hydrophobicity will be taken into account when designing modifications in the amino acid sequence of a zins4 polypeptide, so as not to disrupt the overall structural and biological profile. Of particular interest for replacement are hydrophobic residues selected from the group consisting of Val, Leu and Ile or the group consisting of Met, Gly, Ser, Ala, Tyr and Trp. For example, residues tolerant of substitution could include these hydrophobic residues as shown in SEQ ID NO: 2. Cysteine residues in the cysteine motifs of SEQ ID NO: 2, described herein, will be relatively intolerant of substitution.

The identities of essential amino acids can also be inferred from analysis of sequence similarity between insulin/relaxin family members and zins4. Using methods such as "FASTA" analysis described previously, regions of high similarity are identified within a family of proteins and used to analyze amino acid sequence for conserved regions. An alternative approach to identifying a variant *zins4* polynucleotide on the basis of structure is to determine whether a nucleic acid molecule encoding a potential variant *zins4* polynucleotide can hybridize to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, as discussed above.

Other methods of identifying essential amino acids in the polypeptides of the present invention are procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244:1081 (1989), Bass et al., *Proc. Natl Acad. Sci. USA* 88:4498 (1991), Coombs and Corey, "Site-Directed Mutagenesis and Protein Engineering," in *Proteins: Analysis and Design*, Angeletti (ed.), pages 259-311 (Academic Press, Inc. 1998)). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity as disclosed below to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton et al., *J. Biol. Chem.* 271:4699 (1996).

The present invention also includes functional fragments of zins4 polypeptides and nucleic acid molecules encoding such functional fragments. A "functional" zins4 or fragment thereof defined herein is characterized by its proliferative or differentiating activity, by its ability to induce or inhibit specialized cell functions, or by its ability to bind specifically to an anti-zins4 antibody or zins4 receptor (either soluble or immobilized). As previously described herein, zins4 is characterized by several cleavage sites that generate a number of bioactive zins4

09781077 030904

5

10

20

35

spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., *Biochem.* 30:10832-7, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., *Gene* 46:145, 1986; Ner et al., *DNA* 7:127, 1988).

5 Variants of the disclosed *zins4* DNA and polypeptide sequences can be generated through DNA shuffling as disclosed by Stemmer, *Nature* 370:389-91, 1994, Stemmer, *Proc. Natl. Acad. Sci. USA* 91:10747-51, 1994 and WIPO Publication WO 97/20078. Briefly, variant DNAs are generated by *in vitro* homologous recombination
10 by random fragmentation of a parent DNA followed by reassembly using PCR, resulting in randomly introduced point mutations. This technique can be modified by using a family of parent DNAs, such as allelic variants or DNAs from different species, to introduce additional variability into the process. Selection or screening for the desired activity, followed by additional iterations of mutagenesis and assay provides for
15 rapid "evolution" of sequences by selecting for desirable mutations while simultaneously selecting against detrimental changes.

Mutagenesis methods as disclosed herein can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides in host cells. Mutagenized DNA molecules that encode active
20 polypeptides (e.g., secreted and detected by antibodies, binding assays, or measured by a signal transduction type assay) can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

25 Using the methods discussed herein, one of ordinary skill in the art can identify and/or prepare a variety of polypeptides that are substantially similar to SEQ ID NO:2 or allelic variants thereof and retain the properties of the wild-type protein. For example, using the methods described above, one could identify a receptor binding domain on *zins4*; an extracellular ligand-binding domain of a receptor for *zins4*; other
30 functional or structural domains; affinity tags; or other domains important for protein-protein interactions or signal transduction. Such polypeptides may also include additional polypeptide segments as generally disclosed above.

For any *zins4* polypeptide, including variants and fusion proteins, one of ordinary skill in the art can readily generate a fully degenerate polynucleotide sequence
35 encoding that variant using the information set forth in Tables 1 and 2 above.

Zins4 chimeric molecules are also provided by the invention. *Zins4* in combination with other members of the insulin/relaxin family can be use to form

F06020 2201360

zwitterhormons, proteins having two unrelated biological functions (Bullesbach et al., *Biochem.* 35:9754-60, 1996). Zins4 can be combined with insulin, relaxins, insulin-like growth factors, Leydig factors, Leydig insulin-like peptides, early placenta insulin-like factors, INSL5, INSL6, bombyxins, and their analogs, among others.

5 The zins4 polypeptides of the present invention, including full-length polypeptides, the A chain, the B chain, the C peptide as described herein, biologically active fragments, and fusion polypeptides, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and
10 include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, and
15 Ausubel et al., eds., *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., NY, 1987.

 In general, a DNA sequence encoding a zins4 polypeptide is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will
20 also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of
25 routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

 To insure cleavage of the B and A chains, cells transfected with expression vectors containing DNA sequences encoding zins4 are co-transfected with expression vectors encoding a suitable prohormone convertase, for example furin,
30 PC1/3, PC2, PACE, PACE4, or PC4. Such sequences are known in the art and can be inserted into expression vectors and transfected into cells as described herein.

 To direct a zins4 polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be
35 that of zins4, or may be derived from another secreted protein (e.g., t-PA) or synthesized *de novo*. The secretory signal sequence is operably linked to the zins4 DNA sequence, i.e., the two sequences are joined in the correct reading frame and

positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain secretory signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Alternatively, the secretory signal sequence contained in the polypeptides of the present invention is used to direct other polypeptides into the secretory pathway. The present invention provides for such fusion polypeptides. A signal fusion polypeptide can be made wherein a secretory signal sequence derived from zins4 (amino acid residues 1-25 of SEQ ID NO:2) is operably linked to a DNA sequence encoding another polypeptide using methods known in the art and disclosed herein. The secretory signal sequence contained in the fusion polypeptides of the present invention is preferably fused amino-terminally to an additional peptide to direct the additional peptide into the secretory pathway. Such constructs have numerous applications known in the art. For example, these novel secretory signal sequence fusion constructs can direct the secretion of an active component of a normally non-secreted protein. Such fusions may be used *in vivo* or *in vitro* to direct peptides through the secretory pathway.

Cultured mammalian cells are suitable hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler et al., *Cell* 14:725, 1978; Corsaro and Pearson, *Somatic Cell Genetics* 7:603, 1981; Graham and Van der Eb, *Virology* 52:456, 1973), electroporation (Neumann et al., *EMBO J.* 1:841-5, 1982), DEAE-dextran mediated transfection (Ausubel et al., *ibid.*), and liposome-mediated transfection (Hawley-Nelson et al., *Focus* 15:73, 1993; Ciccarone et al., *Focus* 15:80, 1993, and viral vectors (Miller and Rosman, *BioTechniques* 7:980-90, 1989; Wang and Finer, *Nature Med.* 2:714-6, 1996). The production of recombinant polypeptides in cultured mammalian cells is disclosed, for example, by Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134. Suitable cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573; Graham et al., *J. Gen. Virol.* 36:59-72, 1977) and Chinese hamster ovary (e.g. CHO-K1; ATCC No. CCL 61) cell lines. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Manassas, VA. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See, e.g., U.S. Patent No.

4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978) and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as "stable transfectants." A preferred selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems can also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (e.g. hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used. Alternative markers that introduce an altered phenotype, such as green fluorescent protein, or cell surface proteins such as CD4, CD8, Class I MHC, placental alkaline phosphatase may be used to sort transfected cells from untransfected cells by such means as FACS sorting or magnetic bead separation technology.

Other higher eukaryotic cells can also be used as hosts, including plant cells, insect cells and avian cells. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar et al., *J. Biosci. (Bangalore)* 11:47-58, 1987. Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino et al., U.S. Patent No. 5,162,222 and WIPO publication WO 94/06463. Insect cells can be infected with recombinant baculovirus, commonly derived from *Autographa californica nuclear polyhedrosis virus* (AcNPV). See, King, and Possee, The Baculovirus Expression System: A Laboratory Guide, London, Chapman & Hall; O'Reilly, et al., Baculovirus Expression Vectors: A Laboratory Manual, New York, Oxford University Press., 1994; and, Richardson, C. D., Ed., Baculovirus Expression Protocols. Methods in Molecular Biology, Totowa, NJ, Humana Press, 1995. The second method of making recombinant baculovirus utilizes a transposon-based system described by Luckow (Luckow, et al., *J Virol* 67:4566-79, 1993). This system is sold in the Bac-to-Bac™ kit (Life Technologies, Rockville, MD). This system utilizes a transfer vector, pFastBac1™ (Life Technologies) containing a Tn7 transposon to move the DNA encoding the zins4 polypeptide into a baculovirus genome maintained in *E. coli* as a large plasmid called a

- "bacmid." The pFastBac1™ transfer vector utilizes the AcNPV polyhedrin promoter to drive the expression of the gene of interest, in this case zins4. However, pFastBac1™ can be modified to a considerable degree. The polyhedrin promoter can be removed and substituted with the baculovirus basic protein promoter (also known as *Pcor*, p6.9 or MP promoter) which is expressed earlier in the baculovirus infection, and has been shown to be advantageous for expressing secreted proteins. See, Hill-Perkins and Possee, *J. Gen. Virol.* 71:971-6, 1990; Bonning, et al., *J. Gen. Virol.* 75:1551-6, 1994; and, Chazenbalk and Rapoport, *J. Biol. Chem.* 270:1543-9, 1995. In such transfer vector constructs, a short or long version of the basic protein promoter can be used.
- Moreover, transfer vectors can be constructed which replace the native zins4 secretory signal sequences with secretory signal sequences derived from insect proteins. For example, a secretory signal sequence from Ecdysteroid Glucosyltransferase (EGT), honey bee Melittin (Invitrogen, Carlsbad, CA), or baculovirus gp67 (PharMingen, San Diego, CA) can be used in constructs to replace the native zins4 secretory signal sequence. In addition, transfer vectors can include an in-frame fusion with DNA encoding an epitope tag at the C- or N-terminus of the expressed zins4 polypeptide, for example, a Glu-Glu epitope tag (Grussenmeyer et al., *Proc. Natl. Acad. Sci.* 82:7952-4, 1985). The tag can then be removed after purification. Using a technique known in the art, a transfer vector containing zins4 is transformed into *E. coli*, and screened for bacmids which contain an interrupted lacZ gene indicative of recombinant baculovirus. The bacmid DNA containing the recombinant baculovirus genome is isolated, using common techniques, and used to transfect *Spodoptera frugiperda* cells, e.g. Sf9 cells. Recombinant virus that expresses zins4 is subsequently produced. Recombinant viral stocks are made by methods commonly used the art.
- The recombinant virus is used to infect host cells, typically a cell line derived from the fall armyworm, *Spodoptera frugiperda*. See, in general, Glick and Pasternak, Molecular Biotechnology: Principles and Applications of Recombinant DNA, ASM Press, Washington, D.C., 1994. Another suitable cell line is the High FiveO™ cell line (Invitrogen) derived from *Trichoplusia ni* (U.S. Patent No. 5,300,435). Commercially available serum-free media are used to grow and maintain the cells. Suitable media are Sf900 II™ (Life Technologies) or ESF 921™ (Expression Systems) for the Sf9 cells; and Ex-cello405™ (JRH Biosciences, Lenexa, KS) or Express FiveO™ (Life Technologies) for the *T. ni* cells. The cells are grown up from an inoculation density of approximately $2-5 \times 10^5$ cells to a density of $1-2 \times 10^6$ cells at which time a recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3. Procedures used are generally described in available laboratory manuals (King and Possee, *ibid.*; O'Reilly et al., *ibid.*; Richardson, *ibid.*).

Subsequent purification of the zins4 polypeptide from the supernatant can be achieved using methods described herein.

Fungal cells, including yeast cells, can also be used within the present invention. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Pichia methanolica*. Methods for transforming *S. cerevisiae* cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; and Murray et al., U.S. Patent No. 4,845,075.

Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). A preferred vector system for use in *Saccharomyces cerevisiae* is the *POT1* vector system disclosed by Kawasaki et al. (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media.

Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311; Kingsman et al., U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia methanolica*, *Pichia guilliermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., *J. Gen. Microbiol.* 132:3459-65, 1986 and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells may be utilized according to the methods of McKnight et al., U.S. Patent No. 4,935,349.

Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533.

The use of *Pichia methanolica* as host for the production of recombinant proteins is disclosed in WIPO Publications WO 97/17450, WO 97/17451, WO 98/02536, and WO 98/02565. DNA molecules for use in transforming *P. methanolica* will commonly be prepared as double-stranded, circular plasmids, which are preferably linearized prior to transformation. For polypeptide production in *P. methanolica*, it is preferred that the promoter and terminator in the plasmid be that of a *P. methanolica* gene, such as a *P. methanolica* alcohol utilization gene (*AUG1* or *AUG2*). Other useful promoters include those of the dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes. To facilitate integration of the DNA into the host chromosome, it is preferred to have the entire expression segment of the

0078407 00001

plasmid flanked at both ends by host DNA sequences. A preferred selectable marker for use in *Pichia methanolica* is a *P. methanolica* ADE2 gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21), which allows *ade2* host cells to grow in the absence of adenine. For large-scale, industrial processes where it is desirable to minimize the use of methanol, it is preferred to use host cells in which both methanol utilization genes (*AUG1* and *AUG2*) are deleted. For production of secreted proteins, host cells deficient in vacuolar protease genes (*PEP4* and *PRB1*) are preferred. Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a polypeptide of interest into *P. methanolica* cells. It is preferred to transform *P. methanolica* cells by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant (t) of from 1 to 40 milliseconds, most preferably about 20 milliseconds.

Prokaryotic host cells, including strains of the bacteria *Escherichia coli*, *Bacillus* and other genera are also useful host cells within the present invention. Techniques for transforming these hosts and expressing foreign DNA sequences cloned therein are well known in the art (see, e.g., Sambrook et al., *ibid.*). When expressing a zins4 polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be refolded and dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space and recovering the protein, thereby obviating the need for denaturation and refolding.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-

transfected into the host cell. *P. methanolica* cells are cultured in a medium comprising adequate sources of carbon, nitrogen and trace nutrients at a temperature of about 25°C to 35°C. Liquid cultures are provided with sufficient aeration by conventional means, such as shaking of small flasks or sparging of fermentors. A preferred culture medium

5 for *P. methanolica* is YEPD (2% D-glucose, 2% Bacto™ Peptone (Difco Laboratories, Detroit, MI), 1% Bacto™ yeast extract (Difco Laboratories), 0.004% adenine and 0.006% L-leucine).

It is preferred to purify the polypeptides of the present invention to ≥80% purity, more preferably to ≥90% purity, even more preferably ≥95% purity, and

10 particularly preferred is a pharmaceutically pure state, that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. Preferably, a purified polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin.

Expressed recombinant zins4 polypeptides (or chimeric zins4

15 polypeptides) can be purified using fractionation and/or conventional purification methods and media. Ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps can include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable chromatographic media include derivatized dextrans,

20 agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso

25 Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups,

30 hydroxyl groups and/or carbohydrate moieties. Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers.

35 Methods for binding receptor polypeptides to support media are well known in the art. Selection of a particular method is a matter of routine design and is determined in part

1.06020 22018260

by the properties of the chosen support. See, for example, Affinity Chromatography: Principles & Methods, Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988.

The polypeptides of the present invention can be isolated by exploitation of their structural and biological properties. For example, immobilized metal ion adsorption (IMAC) chromatography can be used to purify histidine-rich proteins, including those comprising polyhistidine tags. Briefly, a gel is first charged with divalent metal ions to form a chelate (Sulkowski, *Trends in Biochem.* 3:1-7, 1985). Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography (*Methods in Enzymol.*, Vol. 182, "Guide to Protein Purification", M. Deutscher, (ed.), Acad. Press, San Diego, 1990, pp.529-39).

Within additional embodiments of the invention, a fusion of the polypeptide of interest and an affinity tag (e.g., HIS tag, Glu-Glu, FLAG, maltose-binding protein, an immunoglobulin domain) may be constructed to facilitate purification. Such fusions include polypeptides comprising affinity tags which further comprise a proteolytic cleavage site between the zins4 polypeptide and the affinity tag. Preferred such sites include thrombin cleavage sites, factor Xa cleavage sites, endoprotease (Arg-Cys or Lys-Cys) sites. To prevent disruption of biological activity, but to facilitate purification, the C peptide can be tagged or replaced.

Additionally, zins4 polypeptides can be purified using prohormone cleavage to isolate the mature protein. The unprocessed polypeptides are first exposed to a prohormone convertase, such as PC1/3, PC3, PC 4, PACE, PACE4, furin, kex-2, to cleave RR or RXXR sites, followed by cleavage of remaining R residues by a carboxypeptidase.

Moreover, using methods described in the art, polypeptide fusions, or hybrid zins4 proteins, are constructed using regions or domains of zins4 in combination with those of paralogs, orthologs, or heterologous proteins (Sambrook et al., *ibid.*, Altschul et al., *ibid.*, Picard *Cur. Opin. Biology* 5:511-5, 1994, and references therein). These methods allow the determination of the biological importance of larger domains or regions in a polypeptide of interest. Such hybrids may alter reaction kinetics, binding, constrict or expand the substrate specificity, or alter tissue and cellular localization of a polypeptide, and can be applied to polypeptides of unknown structure.

Fusion polypeptides can be prepared by methods known to those skilled in the art by preparing each component of the fusion protein and chemically conjugating them. Alternatively, a polynucleotide encoding one or more components of the fusion

protein in the proper reading frame can be generated using known techniques and expressed by the methods described herein. For example, part or all of a domain(s) conferring a biological function may be swapped between zins4 of the present invention with the functionally equivalent domain(s) from another family member. Such domains
 5 include, but are not limited to the secretory signal sequence, the A chain, the B chain and the C peptide, described herein. Such fusion proteins would be expected to have a biological functional profile that is the same or similar to polypeptides of the present invention or other known family proteins or to a heterologous protein, depending on the fusion constructed. Moreover, such fusion proteins may exhibit other properties as
 10 disclosed herein.

Standard molecular biological and cloning techniques can be used to swap the equivalent domains between the zins4 polypeptide and those polypeptides to which they are fused. Generally, a DNA segment that encodes a domain of interest, e.g., a zins4 N-terminal polypeptide, the A chain, the B chain, or the C peptide, or motif
 15 described herein, is operably linked in frame to at least one other DNA segment encoding an additional polypeptide and inserted into an appropriate expression vector, as described herein. Generally DNA constructs are made such that the several DNA segments that encode the corresponding regions of a polypeptide are operably linked in frame to make a single construct that encodes the entire fusion protein, or a functional
 20 portion thereof. For example, a DNA construct would encode from N-terminus to C-terminus a fusion protein comprising a signal polypeptide followed by a mature polypeptide; or a DNA construct would encode from N-terminus to C-terminus a fusion protein comprising a signal polypeptide followed by B chain, C peptide and A chain, or as interchanged with equivalent regions from another protein. Such fusion proteins can
 25 be expressed, isolated, and assayed for activity as described herein.

Protein refolding (and optionally reoxidation) procedures may be advantageously used. zins4 polypeptides or fragments thereof may also be prepared through chemical synthesis. zins4 polypeptides may be monomers or multimers; glycosylated or non-glycosylated; pegylated or non-pegylated; and may or may not
 30 include an initial methionine amino acid residue.

Polypeptides of the present invention can also be synthesized by exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. Methods for synthesizing polypeptides are well known in the art. See, for example, Merrifield, *J. Am. Chem. Soc.* 85:2149, 1963; Kaiser et al.,
 35 *Anal. Biochem.* 34:595, 1970. After the entire synthesis of the desired peptide on a solid support, the peptide-resin is washed with a reagent which cleaves the polypeptide

T06020 2018260

from the resin and removes most of the side-chain protecting groups. Such methods are well established in the art.

Nucleic acid molecules disclosed herein can be used to detect the expression of a *zins4* gene in a biological sample. Such probe molecules include
 5 double-stranded nucleic acid molecules comprising the nucleotide sequences of SEQ ID NO:1, or fragments thereof, as well as single-stranded nucleic acid molecules having the complement of the nucleotide sequences of SEQ ID NO:1, or a fragment thereof. Probe molecules may be DNA, RNA, oligonucleotides, and the like. As an illustration, suitable probes include nucleic acid molecules that bind with a portion of a *zins4* A
 10 chain, B chain or C peptide, or the sequences of SEQ ID Nos: 3, 4, or 5.

In a basic assay, a single-stranded probe molecule is incubated with RNA, isolated from a biological sample, under conditions of temperature and ionic strength that promote base pairing between the probe and target *zins4* RNA species. After separating unbound probe from hybridized molecules, the amount of hybrids is
 15 detected.

Well-established hybridization methods of RNA detection include northern analysis and dot/slot blot hybridization (see, for example, Ausubel *ibid.* and Wu et al. (eds.), "Analysis of Gene Expression at the RNA Level," in Methods in Gene Biotechnology, pages 225-239 (CRC Press, Inc. 1997)). Nucleic acid probes can be
 20 detectably labeled with radioisotopes such as ^{32}P or ^{35}S . Alternatively, *zins4* RNA can be detected with a nonradioactive hybridization method (see, for example, Isaac (ed.), Protocols for Nucleic Acid Analysis by Nonradioactive Probes, Humana Press, Inc., 1993). Typically, nonradioactive detection is achieved by enzymatic conversion of chromogenic or chemiluminescent substrates. Illustrative nonradioactive moieties include
 25 biotin, fluorescein, and digoxigenin.

Zins4 oligonucleotide probes are also useful for *in vivo* diagnosis. As an illustration, ^{18}F -labeled oligonucleotides can be administered to a subject and visualized by positron emission tomography (Tavitian et al., *Nature Medicine* 4:467, 1998).

Numerous diagnostic procedures take advantage of the polymerase chain
 30 reaction (PCR) to increase sensitivity of detection methods. Standard techniques for performing PCR are well-known (see, generally, Mathew (ed.), Protocols in Human Molecular Genetics (Humana Press, Inc. 1991), White (ed.), PCR Protocols: Current Methods and Applications (Humana Press, Inc. 1993), Cotter (ed.), Molecular Diagnosis of Cancer (Humana Press, Inc. 1996), Hanausek and Walaszek (eds.), Tumor
 35 Marker Protocols (Humana Press, Inc. 1998), Lo (ed.), Clinical Applications of PCR (Humana Press, Inc. 1998), and Meltzer (ed.), PCR in Bioanalysis (Humana Press, Inc. 1998)). PCR primers can be designed to amplify a sequence encoding a particular

zins4 domain or motif, such as the A chain, B chain, C peptide, or the sequences of SEQ ID Nos:3, 4, or 5. One variation of PCR for diagnostic assays is reverse transcriptase-PCR (RT-PCR). In the RT-PCR technique, RNA is isolated from a biological sample, reverse transcribed to cDNA, and the cDNA is incubated with *zins4* primers (see, for example, Wu et al. (eds.), "Rapid Isolation of Specific cDNAs or Genes by PCR," in Methods in Gene Biotechnology, CRC Press, Inc., pages 15-28, 1997). PCR is then performed and the products are analyzed using standard techniques.

As an illustration, RNA is isolated from biological sample using, for example, the guanidinium-thiocyanate cell lysis procedure described herein. Alternatively, a solid-phase technique can be used to isolate mRNA from a cell lysate. A reverse transcription reaction can be primed with the isolated RNA using random oligonucleotides, short homopolymers of dT, or *zins4* anti-sense oligomers. Oligo-dT primers offer the advantage that various mRNA nucleotide sequences are amplified that can provide control target sequences. *Zins4* sequences are amplified by the polymerase chain reaction using two flanking oligonucleotide primers that are typically at least 5 bases in length.

PCR amplification products can be detected using a variety of approaches. For example, PCR products can be fractionated by gel electrophoresis, and visualized by ethidium bromide staining. Alternatively, fractionated PCR products can be transferred to a membrane, hybridized with a detectably-labeled *zins4* probe, and examined by autoradiography. Additional alternative approaches include the use of digoxigenin-labeled deoxyribonucleic acid triphosphates to provide chemiluminescence detection, and the C-TRAK colorimetric assay.

Another approach is real time quantitative PCR (Perkin-Elmer Cetus, Norwalk, Ct.). A fluorogenic probe, consisting of an oligonucleotide with both a reporter and a quencher dye attached, anneals specifically between the forward and reverse primers. Using the 5' endonuclease activity of Taq DNA polymerase, the reporter dye is separated from the quencher dye and a sequence-specific signal is generated and increases as amplification increases. The fluorescence intensity can be continuously monitored and quantified during the PCR reaction.

Another approach for detection of *zins4* expression is cycling probe technology (CPT), in which a single-stranded DNA target binds with an excess of DNA-RNA-DNA chimeric probe to form a complex, the RNA portion is cleaved with RNase H, and the presence of cleaved chimeric probe is detected (see, for example, Beggs et al., *J. Clin. Microbiol.* 34:2985, 1996 and Bekkaoui et al., *Biotechniques* 20:240, 1996). Alternative methods for detection of *zins4* sequences can utilize approaches such as nucleic acid sequence-based amplification (NASBA), cooperative

amplification of templates by cross-hybridization (CATCH), and the ligase chain reaction (LCR) (see, for example, Marshall et al., U.S. Patent No. 5,686,272 (1997), Dyer et al., *J. Virol. Methods* 60:161, 1996; Ehricht et al., *Eur. J. Biochem.* 243:358, 1997 and Chadwick et al., *J. Virol. Methods* 70:59, 1998). Other standard methods are
 5 known to those of skill in the art.

Zins4 probes and primers can also be used to detect and to localize *zins4* gene expression in tissue samples. Methods for such *in situ* hybridization are well-known to those of skill in the art (see, for example, Choo (ed.), In Situ Hybridization Protocols, Humana Press, Inc., 1994; Wu et al. (eds.), "Analysis of Cellular DNA or
 10 Abundance of mRNA by Radioactive *In Situ* Hybridization (RISH)," in Methods in Gene Biotechnology, CRC Press, Inc., pages 259-278, 1997 and Wu et al. (eds.), "Localization of DNA or Abundance of mRNA by Fluorescence *In Situ* Hybridization (RISH)," in Methods in Gene Biotechnology, CRC Press, Inc., pages 279-289, 1997).

Various additional diagnostic approaches are well-known to those of
 15 skill in the art (see, for example, Mathew (ed.), Protocols in Human Molecular Genetics Humana Press, Inc., 1991; Coleman and Tsongalis, Molecular Diagnostics, Humana Press, Inc., 1996 and Elles, Molecular Diagnosis of Genetic Diseases, Humana Press, Inc., 1996).

Polypeptides associated with the ovaries are useful for modulating
 20 steroidogenesis, both *in vivo* and *in vitro*, and modulating aspects of the ovarian cycle such as oocyte maturation, ovarian cell-cell interactions, follicular development and rupture, luteal function, promoting uterine implantation of fertilized oocytes as well as modulating activities associated the pregnancy, such as gestation and labor, including the force and rate of contractions. Imbalances in reproductive polypeptides can be used
 25 to assess the existence of diseases, as well as determine whether normal hormonal balance has been restored after administration of a therapeutic agent. Determination of estradiol, progesterone, LH, and FSH, for example, from serum is known by one of skill in the art. Such assays can be used to monitor hormone levels after administration of *zins4 in vivo*, or in a transgenic mouse model where the *zins4* gene is expressed or the
 30 murine ortholog is deleted. *Zins4* polypeptides can be used alone or in combination with other reproductive proteins such as relaxin, as a therapeutic application for pregnancy support.

Proteins of the present invention may also be used in applications for
 enhancing fertilization during assisted reproduction in humans and in animals. Such
 35 assisted reproduction methods are known in the art and include artificial insemination, *in vitro* fertilization, embryo transfer, and gamete intrafallopian transfer. Such methods are useful for assisting those who may have physiological or metabolic disorders that

prevent or impede natural conception. Such methods are also used in animal breeding programs, e.g., for livestock, racehorses, domestic and wild animals, and could be used as methods for the creation of transgenic animals.

Proteins of the present invention may also be useful in therapies for treating reproductive disorders. Disorders such as luteal phase deficiency would benefit from such therapy (Soules, "Luteal phase deficiency: A subtle abnormality of ovulation" in, Infertility: Evaluation and Treatment, Keye et al., eds., Philadelphia, WB Saunders, 1995). Administration of reproductive hormones, such as gonadotropin-releasing hormone, is shown to stimulate reproductive behavior (Riskin and Moss, *Res. Bull.* 11:481-5, 1983; Kadar et al., *Physiol. Behav.* 51:601-5, 1992 and Silver et al., *J. Neuroendocrin.* 4:207-10, 199; King and Millar, *Cell. Mol. Neurobiol.* 15:5-23, 1995). Given the high prevalence of sexual dysfunction and impotence in humans, molecules, polypeptides which may modulate or enhance reproductive activity can find application in developing treatments for these conditions.

Proteins associated with the ovaries may modulate hormones, hormone receptors, growth factors, or cell-cell interactions, of the reproductive cascade or have an involvement in oocyte or ovarian development. The polypeptides, nucleic acid and/or antibodies of the present invention may be used in treatment of disorders associated with gonadal development, pregnancy, pubertal changes, menopause, ovarian cancer, fertility, ovarian function, polycystic ovarian syndrome and other reproductive functions. The molecules of the present invention may used to modulate or to treat or prevent development of pathological conditions in ovary. In particular, certain syndromes or diseases may be amenable to such diagnosis, treatment or prevention. Moreover, natural functions, such as ovulation, may be suppressed or controlled for use in birth control by molecules of the present invention.

Diagnostic methods of the present invention involve the detection of zins4 polypeptides in the serum or tissue biopsy of a patient undergoing analysis of reproductive function or evaluation for possible ovarian cancer. Such polypeptides can be detected using immunoassay techniques and antibodies, described herein, that are capable of recognizing polypeptide epitopes. Such methods include using probes or primers derived, for example, from the nucleotide sequences disclosed herein to detect zins4 expression in a patient sample, such as a blood, saliva, sweat, tissue sample, or the like. For example, probes can be hybridized to tumor tissues and the hybridized complex detected by *in situ* hybridization. *Zins4* sequences can also be detected by PCR amplification using cDNA generated by reverse translation of sample mRNA as a template (PCR Primer A Laboratory Manual, Dieffenbach and Dveksler, eds., Cold Spring Harbor Press, 1995). When compared with a normal control, both increases or

decreases of zins4 expression in a patient sample, relative to that of a control, can be monitored and used as an indicator or diagnostic for disease.

The polypeptides of the present invention may modulate contractility in certain tissues. To identify such activity in zins4, assays known in the art can be applied to tissue samples, such as aortic rings, vas deferens, ileum, uterine and other contractile tissue samples, as well as to organ systems, such as atria, and can be used to determine whether zins4 polypeptide, its agonists or antagonists, enhance or depress contractility. Molecules of the present invention are hence useful for treating dysfunction associated with contractile tissues or can be used to suppress or enhance contractility *in vivo*. As such, molecules of the present invention have utility in treating cardiovascular disease, infertility, *in vitro* fertilization, birth control, treating impotence or other male reproductive dysfunction, as well as inducing birth.

The effect of the zins4 polypeptides, antagonists and agonists of the present invention on contractility of tissues can be measured in a tensiometer that measures contractility and relaxation in tissues. See, Dainty et al., *J. Pharmacol.* 100:767, 1990; Rhee et al., *Neurotox.* 16: 179, 1995; Anderson, *Endocrinol.* 114:364-8, 1984; and Downing and Sherwood, *Endocrinol.* 116:1206-14, 1985. For example, measuring vasodilatation of aortic rings is well known in the art. Briefly, aortic rings are taken from 4 month old Sprague Dawley rats and placed in a buffer solution, such as modified Krebs solution (118.5 mM NaCl, 4.6 mM KCl, 1.2 mM MgSO₄·7H₂O, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂·2H₂O, 24.8 mM NaHCO₃ and 10 mM glucose). One of skill in the art would recognize that this method can be used with other animals, such as rabbits, other rat strains, Guinea pigs, and the like. The rings are then attached to an isometric force transducer (Radnoti Inc., Monrovia, CA) and the data recorded with a Ponemah physiology platform (Gould Instrument systems, Inc., Valley View, OH) and placed in an oxygenated (95% O₂, 5% CO₂) tissue bath containing the buffer solution. The tissues are adjusted to 1 gram resting tension and allowed to stabilize for about one hour before testing. The integrity of the rings can be tested with norepinephrin (Sigma Co., St. Louis, MO) and Carbachol, a muscarinic acetylcholine agonist (Sigma Co.). After integrity is checked, the rings are washed three times with fresh buffer and allowed to rest for about one hour. To test a sample for vasodilatation, or relaxation of the aortic ring tissue, the rings are contracted to two grams tension and allowed to stabilize for fifteen minutes. A zins4 polypeptide sample is then added to 1, 2 or 3 of the 4 baths, without flushing, and tension on the rings recorded and compared to the control rings containing buffer only. Enhancement or relaxation of contractility by zins4 polypeptides, their agonists and antagonists is directly measured by this method, and it can be applied to other contractile tissues such as uterus, prostate, and testis.

09781077-020001

5

10

10

30

35

ovarian cells in culture, and may also prove useful in the study of the ovarian cycle, reproductive function, ovarian cell-cell interactions, and fertilization. Additionally the molecules of the present invention may be useful for promoting the growth and/or development of myocytes in culture, such as cardiac myocytes or myoblasts; skeletal
5 myocytes or myoblasts and smooth muscle cells; chondrocytes; endothelial cells; adipocytes and osteoblasts *in vitro*. For example, molecules of the present invention are useful as components of defined cell culture media, and may be used alone or in combination with other cytokines and hormones to replace serum that is commonly used in cell culture, and may also prove useful in the study of cardiac myocyte
10 hyperplasia and regeneration.

Proteins of the present invention are useful for example, in treating reproductive, prostate, heart, kidney and other disorders, and can be measured *in vitro* using cultured cells or *in vivo* by administering molecules of the present invention to the appropriate animal model. An *in vivo* approach for assaying proteins of the present
15 invention involves viral delivery systems. Exemplary viruses for this purpose include adenovirus, herpesvirus, retroviruses, vaccinia virus, and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer vector for delivery of heterologous nucleic acid (for review, see Becker et al., *Meth. Cell Biol.* 43:161-89, 1994; and Douglas and Curiel, *Science & Medicine* 4:44-
20 53, 1997). The adenovirus system offers several advantages: (i) adenovirus can accommodate relatively large DNA inserts; (ii) can be grown to high-titer; (iii) infect a broad range of mammalian cell types; and (iv) can be used with many different promoters including ubiquitous, tissue specific, and regulatable promoters. Also, because adenoviruses are stable in the bloodstream, they can be administered by
25 intravenous injection.

Using adenovirus vectors where portions of the adenovirus genome are deleted, inserts are incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. In an exemplary system, the essential E1 gene has been deleted from the viral vector, and the virus will not replicate unless the
30 E1 gene is provided by the host cell (the human 293 cell line is exemplary). When intravenously administered to intact animals, adenovirus primarily targets the liver. If the adenoviral delivery system has an E1 gene deletion, the virus cannot replicate in the host cells. However, the host's tissue (e.g., liver) will express and process (and, if a secretory signal sequence is present, secrete) the heterologous protein. Secreted
35 proteins will enter the circulation in the highly vascularized liver, and effects on the infected animal can be determined.

Moreover, adenoviral vectors containing various deletions of viral genes can be used in an attempt to reduce or eliminate immune responses to the vector. Such adenoviruses are E1 deleted, and in addition contain deletions of E2A or E4 (Lusky, et al., *J. Virol.* 72:2022-32, 1998; Raper, et al., *Human Gene Therapy* 9:671-9, 1998). In addition, deletion of E2b is reported to reduce immune responses (Amalfitano, et al., *J. Virol.* 72:926-33, 1998). Moreover, by deleting the entire adenovirus genome, very large inserts of heterologous DNA can be accommodated. Generation of so called "gutless" adenoviruses where all viral genes are deleted are particularly advantageous for insertion of large inserts of heterologous DNA. For review, see Yeh and Perricaudet., *FASEB J.* 11:615-23, 1997.

The adenovirus system can also be used for protein production *in vitro*. By culturing adenovirus-infected non-293 cells under conditions where the cells are not rapidly dividing, the cells can produce proteins for extended periods of time. For instance, BHK cells are grown to confluence in cell factories, then exposed to the adenoviral vector encoding the secreted protein of interest. The cells are then grown under serum-free conditions, which allows infected cells to survive for several weeks without significant cell division. Alternatively, adenovirus vector infected 293 cells can be grown as adherent cells or in suspension culture at relatively high cell density to produce significant amounts of protein (See Garnier et al., *Cytotechnol.* 15:145-55, 1994). With either protocol, an expressed, secreted heterologous protein can be repeatedly isolated from the cell culture supernatant, lysate, or membrane fractions depending on the disposition of the expressed protein in the cell. Within the infected 293 cell production protocol, non-secreted proteins may also be effectively obtained.

As a ligand, the activity of zins4 polypeptide can be measured by a silicon-based biosensor microphysiometer which measures the extracellular acidification rate or proton excretion associated with receptor binding and subsequent physiologic cellular responses. An exemplary device is the Cytosensor™ Microphysiometer manufactured by Molecular Devices, Sunnyvale, CA. A variety of cellular responses, such as cell proliferation, ion transport, energy production, inflammatory response, regulatory and receptor activation, and the like, can be measured by this method. See, for example, McConnell et al., *Science* 257:1906-12, 1992; Pitchford et al., *Meth. Enzymol.* 228:84-108, 1997; Arimilli et al., *J. Immunol. Meth.* 212:49-59, 1998; Van Liefde et al., *Eur. J. Pharmacol.* 346:87-95, 1998. The microphysiometer can be used for assaying adherent or non-adherent eukaryotic or prokaryotic cells. By measuring extracellular acidification changes in cell media over time, the microphysiometer directly measures cellular responses to various stimuli, including zins4 polypeptide, its agonists, or antagonists. Preferably, the

Moreover, zins4 can be used to identify cells, tissues, or cell lines which
30 respond to a zins4-stimulated pathway. The microphysiometer, described above, can be
used to rapidly identify ligand-responsive cells, such as cells responsive to zins4 of the
present invention. Cells can be cultured in the presence or absence of zins4
polypeptide. Those cells which elicit a measurable change in extracellular acidification
in the presence of zins4 are responsive to zins4. Such cell lines, can be used to identify
35 antagonists and agonists of zins4 polypeptide as described above.

35 antagonists and agonists of zins4 polypeptide as described above.

enormous potential in both *in vitro* and *in vivo* applications. For example, zins4 polypeptide and agonist compounds are useful as components of defined cell culture media, and may be used alone or in combination with cytokines and hormones to replace serum that is commonly used in cell culture. Agonists are thus useful in specifically promoting the growth and/or development of mammalian cells *in vitro*, particularly of those derived from reproductive tissues. As such, zins4 polypeptides or agonists are added to tissue culture media for these cell types.

Zins4 can also be used to identify inhibitors (antagonists) of its activity. Test compounds are added to assays disclosed herein to identify compounds that inhibit the activity of zins4. In addition to those assays disclosed herein, samples can be tested for inhibition of zins4 activity within a variety of assays designed to measure receptor binding or the stimulation/inhibition of zins4-dependent cellular responses. For example, zins4-responsive cell lines can be transfected with a reporter gene construct that is responsive to a zins4-stimulated cellular pathway. Reporter gene constructs of this type are known in the art, and will generally comprise a zins4-DNA response element operably linked to a gene encoding an assayable protein, such as luciferase. DNA response elements can include, but are not limited to, cyclic AMP response elements (CRE), hormone response elements (HRE) insulin response element (IRE) (Nasrin et al., *Proc. Natl. Acad. Sci. USA* 87:5273-7, 1990) and serum response elements (SRE) (Shaw et al. *Cell* 56: 563-72, 1989). Cyclic AMP response elements are reviewed in Roestler et al., *J. Biol. Chem.* 263 (19):9063-6; 1988 and Habener, *Molec. Endocrinol.* 4 (8):1087-94; 1990. Hormone response elements are reviewed in Beato, *Cell* 56:335-44; 1989. Candidate compounds, solutions, mixtures or extracts are tested for the ability to inhibit the activity of zins4 on the target cells as evidenced by a decrease in zins4 stimulation of reporter gene expression. Assays of this type will detect compounds that directly block zins4 binding to cell-surface receptors, as well as compounds that block processes in the cellular pathway subsequent to receptor-ligand binding. In the alternative, compounds or other samples can be tested for direct blocking of zins4 binding to receptor using zins4 tagged with a detectable label (e.g., ¹²⁵I, biotin, horseradish peroxidase, FITC, and the like). Within assays of this type, the ability of a test sample to inhibit the binding of labeled zins4 to the receptor is indicative of inhibitory activity, which can be confirmed through secondary assays. Receptors used within binding assays may be cellular receptors or isolated, immobilized receptors.

A zins4 ligand-binding polypeptide can also be used for purification of ligand. The polypeptide is immobilized on a solid support, such as agarose beads, cross-linked agarose, glass, cellulosic resins, silica-based resins, polystyrene, cross-

linked polyacrylamide, or like materials that are stable under the conditions of use. Methods for linking polypeptides to solid supports are known in the art, and include amine chemistry, cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, and hydrazide activation. The resulting
 5 medium will generally be configured in the form of a column, and fluids containing ligand are passed through the column one or more times to allow ligand to bind to the receptor polypeptide. The ligand is then eluted using changes in salt concentration, chaotropic agents (guanidine HCl), or pH to disrupt ligand-receptor binding.

An assay system that uses a ligand-binding receptor (or an antibody, one
 10 member of a complement/ anti-complement pair) or a binding fragment thereof, and a commercially available biosensor instrument (BIAcore, Pharmacia Biosensor, Piscataway, NJ) may be advantageously employed. Such receptor, antibody, member of a complement/anti-complement pair or fragment is immobilized onto the surface of a receptor chip. Use of this instrument is disclosed by Karlsson, *J. Immunol. Methods*
 15 *145:229-40*, 1991 and Cunningham and Wells, *J. Mol. Biol.* *234:554-63*, 1993. A receptor, antibody, member or fragment is covalently attached, using amine or sulfhydryl chemistry, to dextran fibers that are attached to gold film within the flow cell. A test sample is passed through the cell. If a ligand, epitope, or opposite member of the complement/anti-complement pair is present in the sample, it will bind to the
 20 immobilized receptor, antibody or member, respectively, causing a change in the refractive index of the medium, which is detected as a change in surface plasmon resonance of the gold film. This system allows the determination of on- and off-rates, from which binding affinity can be calculated, and assessment of stoichiometry of binding.

25 Ligand-binding receptor polypeptides can also be used within other assay systems known in the art. Such systems include Scatchard analysis for determination of binding affinity (see Scatchard, *Ann. NY Acad. Sci.* *51: 660-72*, 1949) and calorimetric assays (Cunningham et al., *Science* *253:545-48*, 1991; Cunningham et al., *Science* *245:821-25*, 1991).

30 Zins4 polypeptides can also be used to prepare antibodies that bind to zins4 epitopes, peptides or polypeptides. The zins4 polypeptide or a fragment thereof serves as an antigen (immunogen) to inoculate an animal and elicit an immune response. One of skill in the art would recognize that antigenic, epitope-bearing polypeptides contain a sequence of at least 6, preferably at least 9, and more preferably
 35 at least 15 to about 30 contiguous amino acid residues of a zins4 polypeptide (e.g., SEQ ID NO:2). Polypeptides comprising a larger portion of a zins4 polypeptide, i.e., from 10 to 30 residues up to the entire length of the amino acid sequence are included.

As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from inoculating a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats with a zins4 polypeptide or a fragment thereof. The immunogenicity of a zins4 polypeptide may be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of zins4 or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "haptten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments, such as F(ab')₂ and Fab proteolytic fragments. Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides, are also included. Non-human antibodies may be humanized by grafting non-human CDRs onto human framework and constant regions, or by incorporating the entire non-

5

10

25

30

35

1991, also see, Bird et al., Science 242:423, 1988, Ladner et al., U.S. Patent No. 4,946,778, Pack et al., Bio/Technology 11:1271, 1993, and Sandhu, *ibid.*

As an illustration, a scFV can be obtained by exposing lymphocytes to zins4 polypeptide *in vitro*, and selecting antibody display libraries in phage or similar
5 vectors (for instance, through use of immobilized or labeled zins4 protein or peptide). Genes encoding polypeptides having potential zins4 polypeptide binding domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide sequences encoding the polypeptides can be
10 obtained in a number of ways, such as through random mutagenesis and random polynucleotide synthesis. These random peptide display libraries can be used to screen for peptides which interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances. Techniques for creating and screening such random peptide display libraries are known in the art (Ladner et al., U.S. Patent No. 5,223,409, Ladner et al., U.S. Patent No. 4,946,778, Ladner et al., U.S. Patent No. 5,403,484, Ladner et al., U.S. Patent No. 5,571,698, and Kay et al., Phage Display of Peptides and Proteins (Academic Press, Inc. 1996)) and random peptide display libraries and kits for screening such libraries are available commercially, for instance from Clontech (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA),
20 and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened using the zins4 sequences disclosed herein to identify proteins which bind to zins4.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells (see, for example, Larrick *et al.*, Methods: A Companion to Methods in Enzymology 2:106, 1991), Courtenay-Luck, "Genetic Manipulation of Monoclonal Antibodies," in Monoclonal Antibodies: Production, Engineering and Clinical Application, Ritter et al. (eds.), page 166 (Cambridge University Press, 1995), and Ward et al., "Genetic Manipulation and Expression of Antibodies," in Monoclonal Antibodies: Principles and Applications, Birch et al., (eds.), page 137 (Wiley-Liss, Inc. 1995)).

Alternative techniques for generating or selecting antibodies useful
35 herein include *in vitro* exposure of lymphocytes to zins4 protein or peptide, and
selection of antibody display libraries in phage or similar vectors (for instance, through
use of immobilized or labeled zins4 protein or peptide). Genes encoding polypeptides

having potential zins4 polypeptide binding domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide sequences encoding the polypeptides can be obtained in a number of ways, such as through random mutagenesis and random polynucleotide synthesis.

5 These random peptide display libraries can be used to screen for peptides which interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances. Techniques for creating and screening such random peptide display libraries are known in the art (Ladner et al., US Patent NO. 5,223,409; Ladner et al., US Patent NO.

10 4,946,778; Ladner et al., US Patent NO. 5,403,484 and Ladner et al., US Patent NO. 5,571,698) and random peptide display libraries and kits for screening such libraries are available commercially, for instance from Clontech (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA) and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened

15 using the zins4 sequences disclosed herein to identify proteins which bind to zins4. These "binding polypeptides" which interact with zins4 polypeptides can be used for tagging cells; for isolating homolog polypeptides by affinity purification; they can be directly or indirectly conjugated to drugs, toxins, radionuclides and the like. These binding polypeptides can also be used in analytical methods such as for screening

20 expression libraries and neutralizing activity, e.g., for blocking interaction between ligand and receptor, or viral binding to a receptor. The binding polypeptides can also be used for diagnostic assays for determining circulating levels of zins4 polypeptides; for detecting or quantitating soluble zins4 polypeptides as marker of underlying pathology or disease. These binding polypeptides can also act as zins4 "antagonists" to

25 block zins4 binding and signal transduction *in vitro* and *in vivo*. These anti-zins4 binding polypeptides would be useful for inhibiting zins4 activity or protein-binding.

Antibodies are considered to be specifically binding if: 1) they exhibit a threshold level of binding activity, and 2) they do not significantly cross-react with related polypeptide molecules. A threshold level of binding is determined if anti-zins4

30 antibodies herein bind to a zins4 polypeptide, peptide or epitope with an affinity at least 10-fold greater than the binding affinity to control (non-zins4) polypeptide. It is preferred that the antibodies exhibit a binding affinity (K_d) of 10^6 M^{-1} or greater, preferably 10^7 M^{-1} or greater, more preferably 10^8 M^{-1} or greater, and most preferably 10^9 M^{-1} or greater. The binding affinity of an antibody can be readily determined by

35 one of ordinary skill in the art, for example, by Scatchard analysis (Scatchard, G., *Ann. NY Acad. Sci.* 51: 660-672, 1949).

Whether anti-zins4 antibodies do not significantly cross-react with related polypeptide molecules is shown, for example, by the antibody detecting zins4 polypeptide but not known related polypeptides using a standard Western blot analysis (Ausubel et al., *ibid.*). Examples of known related polypeptides are those disclosed in the prior art, such as known orthologs, and paralogs, and similar known members of a protein family. Screening can also be done using non-human zins4, and zins4 mutant polypeptides. Moreover, antibodies can be "screened against" known related polypeptides, to isolate a population that specifically binds to the zins4 polypeptides. For example, antibodies raised to zins4 are adsorbed to related polypeptides adhered to insoluble matrix; antibodies specific to zins4 will flow through the matrix under the proper buffer conditions. Screening allows isolation of polyclonal and monoclonal antibodies non-crossreactive to known closely related polypeptides (Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988; Current Protocols in Immunology, Cooligan, et al. (eds.), National Institutes of Health, John Wiley and Sons, Inc., 1995). Screening and isolation of specific antibodies is well known in the art. See, Fundamental Immunology, Paul (eds.), Raven Press, 1993; Getzoff et al., *Adv. in Immunol.* 43: 1-98, 1988; Monoclonal Antibodies: Principles and Practice, Goding, J.W. (eds.), Academic Press Ltd., 1996; Benjamin et al., *Ann. Rev. Immunol.* 2: 67-101, 1984. Specifically binding anti-zins4 antibodies can be detected by a number of methods in the art, and disclosed below.

A variety of assays known to those skilled in the art can be utilized to detect antibodies which bind to zins4 proteins or polypeptides. Exemplary assays are described in detail in Antibodies: A Laboratory Manual, Harlow and Lane (Eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmunoassay, radioimmuno-precipitation, enzyme-linked immunosorbent assay (ELISA), dot blot or Western blot assay, inhibition or competition assay, and sandwich assay. In addition, antibodies can be screened for binding to wild-type versus mutant zins4 protein or polypeptide.

Antibodies to zins4 may be used for tagging cells that express zins4; for isolating zins4 by affinity purification; for diagnostic assays for determining circulating levels of zins4 polypeptides; for detecting or quantitating soluble zins4 as marker of underlying pathology or disease; in analytical methods employing FACS; for screening expression libraries; for generating anti-idiotypic antibodies; and as neutralizing antibodies or as antagonists to block zins4 activity *in vitro* and *in vivo*. Suitable direct tags or labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like; indirect tags or labels may feature use of biotin-avidin or other complement/anti-

complement pairs as intermediates. Antibodies herein may also be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications. Moreover, antibodies to zins4 or fragments thereof may be used *in vitro* to detect denatured zins4 or fragments thereof in assays, for example, Western Blots or other assays known in the art.

Antibodies or polypeptides herein can also be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications. For instance, polypeptides or antibodies of the present invention can be used to identify or treat tissues or organs that express a corresponding anti-complementary molecule (receptor or antigen, respectively, for instance). More specifically, zins4 polypeptides or anti-zins4 antibodies, or bioactive fragments or portions thereof, can be coupled to detectable or cytotoxic molecules and delivered to a mammal having cells, tissues or organs that express the anti-complementary molecule.

Suitable detectable molecules may be directly or indirectly attached to the polypeptide or antibody, and include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like. Suitable cytotoxic molecules may be directly or indirectly attached to the polypeptide or antibody, and include bacterial or plant toxins (for instance, diphtheria toxin, *Pseudomonas* exotoxin, ricin, abrin and the like), as well as therapeutic radionuclides, such as iodine-131, rhenium-188 or yttrium-90 (either directly attached to the polypeptide or antibody, or indirectly attached through means of a chelating moiety, for instance). Polypeptides or antibodies may also be conjugated to cytotoxic drugs, such as adriamycin. For indirect attachment of a detectable or cytotoxic molecule, the detectable or cytotoxic molecule can be conjugated with a member of a complementary/ anticomplementary pair, where the other member is bound to the polypeptide or antibody portion. For these purposes, biotin/streptavidin is an exemplary complementary/ anticomplementary pair.

In another embodiment, polypeptide-toxin fusion proteins or antibody-toxin fusion proteins can be used for targeted cell or tissue inhibition or ablation (for instance, to treat cancer cells or tissues). Alternatively, if the polypeptide has multiple functional domains (i.e., an activation domain or a receptor binding domain, plus a targeting domain), a fusion protein including only the targeting domain may be suitable for directing a detectable molecule, a cytotoxic molecule or a complementary molecule to a cell or tissue type of interest. In instances where the domain only fusion protein includes a complementary molecule, the anti-complementary molecule can be conjugated to a detectable or cytotoxic molecule. Such domain-complementary

molecule fusion proteins thus represent a generic targeting vehicle for cell/tissue-specific delivery of generic anti-complementary-detectable/ cytotoxic molecule conjugates.

The bioactive polypeptide or antibody conjugates described herein can be delivered intravenously, intraarterially or intraductally, or may be introduced locally at the intended site of action.

Molecules of the present invention can be used to identify and isolate receptors that bind zins4 polypeptide. For example, proteins and peptides of the present invention can be immobilized on a column and membrane preparations run over the column (Immobilized Affinity Ligand Techniques, Hermanson et al., eds., Academic Press, San Diego, CA, 1992, pp.195-202). Proteins and peptides can also be radiolabeled (Methods in Enzymol., vol. 182, "Guide to Protein Purification", M. Deutscher, ed., Acad. Press, San Diego, 1990, 721-37) or photoaffinity labeled (Brunner et al., *Ann. Rev. Biochem.* 62:483-514, 1993 and Fedan et al., *Biochem. Pharmacol.* 33:1167-80, 1984) and specific cell-surface proteins can be identified.

The polypeptides, antagonists, agonists, nucleic acid and/or antibodies of the present invention may be used in treatment of disorders associated with gonadal development, pregnancy, pubertal changes, menopause, ovarian cancer, fertility, ovarian function, polycystic ovarian syndrome, uterine cancer, endometriosis, libido, myalgia and neuralgia associated with reproductive phenomena, male sexual dysfunction, impotency, prostate cancer, testicular cancer, and dysfunction. The molecules of the present invention may used to modulate or to treat or prevent development of pathological conditions in such diverse tissue as ovary, heart, testis, and kidney. In particular, certain syndromes or diseases may be amenable to such diagnosis, treatment or prevention. Moreover, natural functions, such as embryo implantation or spermatogenesis, may be suppressed or controlled for use in birth control by molecules of the present invention.

Polynucleotides and polypeptides of the present invention will be useful as educational tools in laboratory practicum kits for courses related to genetics and molecular biology, protein chemistry, and antibody production and analysis. Due to its unique polynucleotide and polypeptide sequences, molecules of zins4 can be used as standards or as "unknowns" for testing purposes. For example, zins4 polynucleotides can be used as an aid, such as, for example, to teach a student how to prepare expression constructs for bacterial, viral, or mammalian expression, including fusion constructs, wherein *zins4* is the gene to be expressed; for determining the restriction endonuclease cleavage sites of the polynucleotides; determining mRNA and DNA localization of *zins4* polynucleotides in tissues (*i.e.*, by northern and Southern blotting

FOUO 20130204

as well as polymerase chain reaction); and for identifying related polynucleotides and polypeptides by nucleic acid hybridization.

Zins4 polypeptides can be used as an aid to teach preparation of antibodies; identifying proteins by western blotting; protein purification; determining
 5 the weight of produced zins4 polypeptides as a ratio to total protein produced; identifying peptide cleavage sites; coupling amino and carboxyl terminal tags; amino acid sequence analysis, as well as, but not limited to monitoring biological activities of both the native and tagged protein *in vitro* and *in vivo*.

Zins4 polypeptides can also be used to teach analytical skills such as
 10 mass spectrometry, circular dichroism to determine conformation, especially of the four alpha helices, x-ray crystallography to determine the three-dimensional structure in atomic detail, nuclear magnetic resonance spectroscopy to reveal the structure of proteins in solution. For example, a kit containing the zins4 can be given to the student to analyze. Since the amino acid sequence would be known by the instructor, the
 15 protein can be given to the student as a test to determine the skills or develop the skills of the student, the instructor would then know whether or not the student has correctly analyzed the polypeptide. Since every polypeptide is unique, the educational utility of zins4 would be unique unto itself.

The antibodies which bind specifically to zins4 can be used as a teaching
 20 aid to instruct students how to prepare affinity chromatography columns to purify zins4, cloning and sequencing the polynucleotide that encodes an antibody and thus as a practicum for teaching a student how to design humanized antibodies. The *zins4* gene, polypeptide, or antibody would then be packaged by reagent companies and sold to educational institutions so that the students gain skill in art of molecular biology.
 25 Because each gene and protein is unique, each gene and protein creates unique challenges and learning experiences for students in a lab practicum. Such educational kits containing the *zins4* gene, polypeptide, or antibody are considered within the scope of the present invention.

Polynucleotides encoding zins4 polypeptides are useful within gene
 30 therapy or gene transfer applications where it is desired to increase or inhibit zins4 activity. If a mammal has a mutated or absent *zins4* gene, the *zins4* gene can be introduced into the cells of the mammal. In one embodiment, a gene encoding a zins4 polypeptide is introduced *in vivo* in a viral vector. Such vectors include an attenuated or defective DNA virus, such as, but not limited to, herpes simplex virus (HSV),
 35 papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. A defective virus is not infective after introduction into a cell. Use of

defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Examples of particular vectors include, but are not limited to, a defective herpes simplex virus 1 (HSV1) vector (Kaplitt et al., *Molec. Cell. Neurosci.* 2:320-30, 1991); an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al., *J. Clin. Invest.* 90:626-30, 1992; and a defective adeno-associated virus vector (Samulski et al., *J. Virol.* 61:3096-101, 1987; Samulski et al., *J. Virol.* 63:3822-8, 1989).

In another embodiment, a *zins4* gene can be introduced in a retroviral vector, e.g., as described in Anderson et al., U.S. Patent No. 5,399,346; Mann et al. *Cell* 33:153, 1983; Temin et al., U.S. Patent No. 4,650,764; Temin et al., U.S. Patent No. 4,980,289; Markowitz et al., *J. Virol.* 62:1120, 1988; Temin et al., U.S. Patent No. 5,124,263; International Patent Publication No. WO 95/07358, published March 16, 1995 by Dougherty et al.; and Kuo et al., *Blood* 82:845, 1993. Alternatively, the vector can be introduced by lipofection *in vivo* using liposomes. Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7, 1987; Mackey et al., *Proc. Natl. Acad. Sci. USA* 85:8027-31, 1988). The use of lipofection to introduce exogenous genes into specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. More particularly, directing transfection to particular cells represents one area of benefit. For instance, directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides (e.g., hormones or neurotransmitters), proteins such as antibodies, or non-peptide molecules can be coupled to liposomes chemically.

It is possible to remove the target cells from the body; to introduce the vector as a naked DNA plasmid; and then to re-implant the transformed cells into the body. Naked DNA vectors for gene therapy or gene transfer can be introduced into the desired host cells by methods known in the art, e.g., transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun or use of a DNA vector transporter. See, e.g., Wu et al., *J. Biol. Chem.* 267:963-7, 1992; Wu et al., *J. Biol. Chem.* 263:14621-4, 1988.

Antisense methodology can be used to inhibit *zins4* gene transcription, such as to inhibit cell proliferation *in vivo*. Polynucleotides that are complementary to a segment of a *zins4*-encoding polynucleotide (e.g., a polynucleotide as set forth in SEQ ID NO:1) are designed to bind to *zins4*-encoding mRNA and to inhibit translation of

such mRNA. Such antisense polynucleotides are used to inhibit expression of *zins4* polypeptide-encoding genes in cell culture or in a subject.

The present invention also provides reagents which will find use in diagnostic applications. For example, the *zins4* gene, a probe comprising *zins4* DNA or RNA or a subsequence thereof can be used to confirm that the *zins4* gene is present on human chromosome 19 and can be used as a marker to determine if a mutation has occurred. *Zins4* is located in the 19p13.11 region of chromosome 19. Detectable chromosomal aberrations at the *zins4* gene locus include, but are not limited to, aneuploidy, gene copy number changes, insertions, deletions, restriction site changes and rearrangements. Such aberrations can be detected using polynucleotides of the present invention by employing molecular genetic techniques, such as restriction fragment length polymorphism (RFLP) analysis, fluorescence *in situ* hybridization methods, short tandem repeat (STR) analysis employing PCR techniques, and other genetic linkage analysis techniques known in the art (Sambrook et al., *ibid.*; Ausubel et al., *ibid.*). Such probes include, but are not limited to, polynucleotides 74-156, 74-159, 74-162, 163-342, 163-345, 163-348, 163-351, 163-354, 355-426, 1-73, 1-162, 1-342, 74-342, 74-345, 74-348, 74-351, and 74-354.

The precise knowledge of a gene's position can be useful for a number of purposes, including: 1) determining if a sequence is part of an existing contig and obtaining additional surrounding genetic sequences in various forms, such as YACs, BACs or cDNA clones; 2) providing a possible candidate gene for an inheritable disease which shows linkage to the same chromosomal region; and 3) cross-referencing model organisms, such as mouse, which may aid in determining what function a particular gene might have.

In general, the diagnostic methods used in genetic linkage analysis, to detect a genetic abnormality or aberration in a patient, are known in the art. Most diagnostic methods comprise the steps of (a) obtaining a genetic sample from a potentially diseased patient, diseased patient or potential non-diseased carrier of a recessive disease allele; (b) producing a first reaction product by incubating the genetic sample with a *zins4* polynucleotide probe wherein the polynucleotide will hybridize to complementary polynucleotide sequence, such as in RFLP analysis or by incubating the genetic sample with sense and antisense primers in a PCR reaction under appropriate PCR reaction conditions; (iii) Visualizing the first reaction product by gel electrophoresis and/or other known method such as visualizing the first reaction product with a *zins4* polynucleotide probe wherein the polynucleotide will hybridize to the complementary polynucleotide sequence of the first reaction; and (iv) comparing the visualized first reaction product to a second control reaction product of a genetic

sample from wild type patient. A difference between the first reaction product and the control reaction product is indicative of a genetic abnormality in the diseased or potentially diseased patient, or the presence of a heterozygous recessive carrier phenotype for a non-diseased patient, or the presence of a genetic defect in a tumor from a diseased patient, or the presence of a genetic abnormality in a fetus or pre-implantation embryo. For example, a difference in restriction fragment pattern, length of PCR products, length of repetitive sequences at the zins4 genetic locus, and the like, are indicative of a genetic abnormality, genetic aberration, or allelic difference in comparison to the normal wild type control. Controls can be from unaffected family members, or unrelated individuals, depending on the test and availability of samples. Genetic samples for use within the present invention include genomic DNA, mRNA, and cDNA isolated from any tissue or other biological sample from a patient, such as but not limited to, blood, saliva, semen, embryonic cells, amniotic fluid, and the like. The polynucleotide probe or primer can be RNA or DNA, and will comprise a portion of SEQ ID NO:1, the complement of SEQ ID NO:1, or an RNA equivalent thereof. Such methods of showing genetic linkage analysis to human disease phenotypes are well known in the art. For reference to PCR based methods in diagnostics see, generally, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), White (ed.), *PCR Protocols: Current Methods and Applications* (Humana Press, Inc. 1993), Cotter (ed.), *Molecular Diagnosis of Cancer* (Humana Press, Inc. 1996), Hanausek and Walaszek (eds.), *Tumor Marker Protocols* (Humana Press, Inc. 1998), Lo (ed.), *Clinical Applications of PCR* (Humana Press, Inc. 1998), and Meltzer (ed.), *PCR in Bioanalysis* (Humana Press, Inc. 1998)).

Aberrations associated with the zins4 locus can be detected using nucleic acid molecules of the present invention by employing standard methods for direct mutation analysis, such as restriction fragment length polymorphism analysis, short tandem repeat analysis employing PCR techniques, amplification-refractory mutation system analysis, single-strand conformation polymorphism detection, RNase cleavage methods, denaturing gradient gel electrophoresis, fluorescence-assisted mismatch analysis, and other genetic analysis techniques known in the art (see, for example, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), Marian, *Chest* 108:255 (1995), Coleman and Tsongalis, *Molecular Diagnostics* (Humana Press, Inc. 1996), Elles (ed.) *Molecular Diagnosis of Genetic Diseases* (Humana Press, Inc. 1996), Landegren (ed.), *Laboratory Protocols for Mutation Detection* (Oxford University Press 1996), Birren *et al.* (eds.), *Genome Analysis, Vol. 2: Detecting Genes* (Cold Spring Harbor Laboratory Press 1998), Dracopoli *et al.* (eds.), *Current Protocols in Human Genetics* (John Wiley & Sons 1998), and Richards and

5

10

20

25

30

35

cDNA can be used to isolate murine zins4 mRNA, cDNA and genomic DNA, which are subsequently used to generate knockout mice. Relaxin knock out mice have been generated (Zhao et al., *Ednocrin.* 140:445-52, 1999). Transgenic mice engineered to over-expresses human polypeptides or mouse polypeptides corresponding to the human A chain, B chain or C peptide, can also be used. These mice may be employed to study the zins4 gene and the protein encoded thereby in an *in vivo* system, and can be used as *in vivo* models for corresponding human diseases. Moreover, transgenic mice expression of zins4 antisense polynucleotides or ribozymes directed against zins4, described herein, can be used analogously to transgenic mice described above.

10 A pharmaceutically effective amount of a zins4 polypeptide of the present invention can be formulated with pharmaceutically acceptable carriers for parenteral, oral, nasal, rectal, topical, transdermal administration or the like, according to conventional methods. Formulations may further include one or more diluents, fillers, emulsifiers, preservatives, buffers, excipients, and the like, and may be provided
15 in such forms as liquids, powders, emulsions, suppositories, liposomes, transdermal patches and tablets, for example. Slow or extended-release delivery systems, including any of a number of biopolymers (biological-based systems), systems employing liposomes, and polymeric delivery systems, can also be utilized with the compositions described herein to provide a continuous or long-term source of the zins4 polypeptide or antagonist. Such slow release systems are applicable to formulations, for example,
20 for oral, topical and parenteral use. The term "pharmaceutically acceptable carrier" refers to a carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredients and which is not toxic to the host or patient. One skilled in the art may formulate the compounds of the present invention in an appropriate manner, and in accordance with accepted practices, such as those disclosed
25 in Remington: The Science and Practice of Pharmacy, Gennaro, ed., Mack Publishing Co., Easton PA, 19th ed., 1995.

As used herein a "pharmaceutically effective amount" of a zins4 polypeptide, agonists or antagonist is an amount sufficient to induce a desired
30 biological result. The result can be alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. For example, an effective amount of a zins4 polypeptide is that which provides either subjective relief of symptoms or an objectively identifiable improvement as noted by the clinician or other qualified observer. Effective amounts of the zins4 polypeptides can vary widely
35 depending on the disease or symptom to be treated. The amount of the polypeptide to be administered and its concentration in the formulations, depends upon the vehicle selected, route of administration, the potency of the particular polypeptide, the clinical

condition of the patient, the side effects and the stability of the compound in the formulation. Thus, the clinician will employ the appropriate preparation containing the appropriate concentration in the formulation, as well as the amount of formulation administered, depending upon clinical experience with the patient in question or with similar patients. Such amounts will depend, in part, on the particular condition to be treated, age, weight, and general health of the patient, and other factors evident to those skilled in the art. Typically a dose will be in the range of 0.1-100 mg/kg of subject. Doses for specific compounds may be determined from *in vitro* or *ex vivo* studies in combination with studies on experimental animals. Concentrations of compounds found to be effective *in vitro* or *ex vivo* provide guidance for animal studies, wherein doses are calculated to provide similar concentrations at the site of action.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

09784077-020904